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MOLECULAR MECHANISMS OF CYTOPATHOGENICITY OF PRIMATE LYMPHOTROPIC RETROVIRUSES: RELEVANCE TO TREATMENT AND VACCINE FOR AIDS

Annual Report

Covering the Period 9/29/87 to 9/28/88

by

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The molecular basis of virus transmission, cytopathogenicity, viral particle formation and genomic activation of HIV-1 was investigated through the use of deletion mutants in the vif, nef, 5' region and LTR. The Biological effects of these alterations were examined following transfection into a variety of cell lines. Mutants with deletions of 37 amino acids at the 3' end of gp41 are capable of producing infectious virus particles and of transmitting virus to H9 cells. The last 6 amino acids residues of gp41 appear to be important in cell killing, while the last 17 amino acid residues appear to be important for cell to cell transmission. The cytopathic effect of the C-terminal gp41 deletion mutants equaled that of the wild-type virus. Therefore, these studies suggest that caution should be used in selecting candidate immunogens for vaccination derived from the HIV env region.

The role of tat in HIV activation was examined by use of a microscale protein binding assay. The detection of a specific 68 KD enhancer binding protein produced in response to

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PHA-stimulation of Jurkat cells has suggested that this protein may be involved in the enhancement of HIV-LTR mediated transcription. A 30-fold increase in the synthesis of CAT was observed when tat was injected with the TAR-CAT transcripts. These results provide direct evidence for a post-transcriptional mechanism for transactivation by tat of genes linked to the TAR region of HIV.

Genetic comparisons of HIV-2, SIV, and HIV-1 revealed that all three retroviruses share common biological features and genetic structure. Equivalent open reading frames are observed between all three retroviruses with a few differences. The presence of an additional gene was noted in HIV-2, SIV_{mac}, and SIV_{agm}, when compared to HIV-1. The expression of this gene in HIV-2 infected individuals and infected cell in vitro were studied. A 16Kd protein was found in HIV-2 infected cells which reacted to rabbit antiserum for the recombinant vpx protein. The vpx protein was localized in the cytoplasm of infected cells and was found in the mature virion. A second difference between HIV-2/SIV and HIV-1 was the smaller size of the transmembrane protein. Two synthetic peptides were generated from the inferred amino acid sequence of SIV_{mac} for this unexpressed region. The protein synthesized from this open reading frame is expressed in vivo. No correlation was found to exist between the expression of the total transmembrane gene and the cytopathogenicity. In order to establish an animal model for vaccine testing, the functions of the accessory and structural genes in HIV-2 are being investigated in vivo. A biologically active clone of HIV-2 has been obtained, characterized, and used to infect rhesus macaques. Characterization of the virus obtained from the monkeys should shed some light on changes which occur in vivo during infection.

I. SUMMARY

The biological parameters of HIV infection were examined in an in vitro model system for AIDS in which the molecular basis of viral gene functions could be dissected. Recombinant DNA techniques were used to generate a series of mutations in the non-structural regions of the viral genome and the biological effects of these alterations were examined following transfection into Cos-1 cells or a variety of lymphoid cell lines. Viral functions were examined at the level of virus transmission, cytopathogenicity, infectious particle formation, and genomic activation.

Studies of the biological activity of mutations in the 3' env/nef region have shown that mutants of up to 37 amino acids deleted from the 3' end of the gp41 are still capable of producing infectious virus particles and of transmitting virus to H9 cells. However, mutants with deletions of 15 or more amino acids can not propagate in Molt-3 cells.

An area important in cell killing lies in the last 6 amino acid residues of gp41. The cytopathic effect of the C-terminal gp41 deletion mutants in coculture far exceeded that of cell-free virions, and equaled that of wild-type virus. These studies therefore suggest that caution should be used in selecting candidate immunogens for vaccination derived from the HIV env region, since they might themselves be cytopathic for susceptible cells.

The mechanism of virus transmission by mutants in vif has been studied with the aid of specific antisera to cell and viral determinants. The differential effects of various human sera to block either the cell-free or cell-associated transmission route suggest that different epitopes are involved in these modes of transmission.

Characterization of deletion mutants at the 5' end of the HIV genome have identified two mutants, Δ293 and #79 which synthesize all classes of viral mRNA, but the decrease in infectivity and viral RNA content of virions suggest a possible defect in packaging.

A microscale protein binding assay was designed to study the involvement of other viral and cellular factors/proteins which play a role in HIV activation by tat. Oligonucleotides containing the enhancer element of the HIV LTR were synthesized and reacted with ^{35}S-methionine labeled cell extracts of a variety of cells before and after stimulation with PHA. A specific enhancer binding protein (HIVEN86A) present in H9 cells and produced in response to PHA-stimulation of Jurkat cells has suggested that this protein may be involved in the enhancement of HIV-LTR mediated transcription. The fact

that the same 86 kd protein is detected in a kappa-immunoglobulin producing cell line suggests HIVEN86A is a candidate for NF-KB activity.

An in vitro system for examining the post-transcriptional effects of tat was designed, which would allow us to study these mechanisms in more detail. Transcripts containing the HIV-1 LTR linked to the chloramphenicol amino transferase (CAT) gene were generated and used for the examination of the post-transcriptional effects of tat. These transcripts were microinjected in Xenopus oocytes along with plasmids containing the tat gene. A 30-fold increase in the amount of CAT synthesized was observed when tat was injected simultaneously with the TAR-CAT transcripts, than when the tat gene was left out. These results provide direct evidence that transactivation by tat of genes linked to the HIV-LTR is at least in part post-transcriptional.

A genetic comparison of HIV-2 and SIV_{mac} to HIV-1 revealed that all three retroviruses share common biological and genetic features. Equivalent open reading frames corresponding to most of the HIV-1 genes were observed in HIV-2 and SIV_{mac}. Two differences were noted, however. An additional gene (vpx) was found in HIV-2 and SIV_{mac} but not in HIV-1. We decided to study the expression of this gene in HIV-2 infected individuals and in cells. A recombinant protein was generated and used in an immunoblot assay. A 16 kD protein was detected in HIV-2 infected cells and was found to be localized in the cytoplasm and in mature virions. A second difference between HIV-2 and HIV-1 was the smaller size of the transmembrane protein. Two synthetic peptides to the non-expressed region were generated and used in Western blots using sera of SIV infected monkeys and HIV-2 infected humans. This region was found to be expressed in vivo, but this expression did not correlate with pathogenicity.

Since HIV-2 can infect monkeys, studies can be conducted to determine the mutation rate in vivo using a cloned isolate. A biologically active clone of HIV-2 has been obtained and characterized during the past year. Studies are now in progress to examine the changes in the HIV-2 genome which occur in vivo in infected monkeys.

II. FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the

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III. BODY OF THE REPORT

A) STATEMENT OF PROBLEM

The rapid spread of AIDS in the U.S. and worldwide has led to extensive efforts to develop therapy regimens for those already infected with HIV and to develop vaccines to protect populations at risk for the disease. Standard approaches to vaccine development, such as use of attenuated or inactivated viruses has been considered potentially too risky because of the possibility that even low levels of incompletely inactivated virus may be capable of initiating productive infection and leading to disease. Most efforts have therefore concentrated on the production of either natural or recombinant subunit vaccines which would have no possibility of containing infectious particles (1, 2, 3). Initial efforts along these lines, however, have met with very limited success due to the extensive variability of the viral genome and the complexity of viral functions expressed in latent and productive infections (4).

Although still not fully satisfactory, somewhat better success has been realized in developing therapeutic agents for blocking the progression of the disease in individuals already infected with HIV. The most successful therapy thus far has been with drugs, such as AZT, which interfere with viral reverse transcriptase activity (5). Although these drugs are effective in preventing the expression of full scale infection, they can be somewhat toxic and are very expensive. Furthermore, these drugs really do not "cure" AIDS but merely keep it in check. The ability of HIV-1 to integrate into the host genome as a provirus allows it to remain quiescent or maintain only very low levels of expression for long periods of time. When treatment is discontinued, the virus can become reactivated leading to a resumption of progression to disease.

A clear understanding of the biological complexities of HIV infections and the molecular basis of cytopathogenicity will provide a valuable tool in designing systematic approaches to the development of appropriate vaccines and therapies for combating AIDS. Our approach to this problem was to examine the biological parameters of infection by various natural isolates of HIV-1 and HIV-2 in a cell culture system which provided a model system for AIDS. The viral determinants for the various activities was correlated to the DNA sequence of the viruses. This approach was made possible by use recombinant DNA techniques to generate a series of deletion mutants in the non-structural portions of the viral genome and to examine the resulting biological effects on virus transmission, cytopathogenicity, formation of infectious particles and genomic activation upon infection into permissive host cells. The results of these studies have

shown that HIV-1 is far more complicated than originally believed, being subject to very complex regulatory mechanisms and expressing a wide range of biological activities. The current work has been divided into 3 major sections. Mutation studies of the nef, vif and 5' regions of the virus have been aimed at examining the molecular basis of transmission, cell killing and production of infectious particles (Section 1). The regulation of virus expression by the tat gene product and its interactions with the viral LTR and cellular factors was examined in Section 2. In Section 3, the relationship between different isolates of HIV-1, HIV-2, ansd SIV were compared in terms of DNA sequence, genome organization, and biological activcity. A clear understanding of the functions of HIV and the molecular basis of these activities will provide valuable insight into planning vaccine and therapeutic approaches to blocking HIV infections.

B) BACKGROUND

The human immune deficiency virus (HIV), a cytopathic retrovirus, is implicated to be the causative agent of the acquired immune deficiency syndrome (AIDS), by seroepidemiology, virus isolation, and molecular epidemiology (6). Infection with HIV-1 in vivo is usually associated with an asymptomatic interval which frequently lasts from several weeks to several years followed by a progressive impairment of the immune functions, and finally leading to the development of actual disease (7). HIV-1 virus selectively infects and kills OKT4+ helper/inducer lymphocytes which play a critical role in the regulation of the immune response (8). Viral replication involves the formation of and intermediat "provirus" stage in which a double stranded copy of the viral genome integrates into the host chromosome. This provirus can then remain in an inactive, quiescent stage or serve as the source of active viral replication following activation. The selective depletion of the OKT4 cells in the peripheral blood upon activation of the viral genome apparently leads to the observed immunodeficiency in the latter stages of the disease, such that the infected individual becomes susceptible to opportunistic infections and dies. The viral determinants of infectious particle assembly, cell killing, transmission of infectious virions and regulation of viral expression have not been fully characterized. A closer examination of these viral functions will be valuable in designing approaches to blocking infections with HIV.

The molecular basis of HIV-1 infection can be studied in an in vitro model system for AIDS, in which the virus can be propagated in PHA stimulated peripheral blood lymphocytes (PBL's) or in a variety of lymphoid cell lines (9). Molecularly cloned

proviral DNA can be transfected into these cultured cells to generate infectious virions and cytopathic effects in vitro which are indistinguishable from virus obtained by cocultivation of permissive cells with the PBL's of AIDS patients (10). Such studies with a cloned viral DNA have provided direct evidence that a product of the HIV genome mediates cell killing. Genetic manipulation to alter defined portions of the HIV-1 DNA clone using standard recombinant DNA techniques will allow us to dissect the viral determinants of pathogenicity, transmission, and other viral functions.

Extensive immunologic, genetic and molecular analysis of the HIV-1 retrovirus in the last few years has shown the viral genome to be far more complex than that of most known retroviruses. In addition to the structural genes for the core proteins (gag), the viral enzymes (pol), and the envelope glycoproteins (env), several accessory genes have been identified either serologically or as open reading frames in the viral DNA (Table 1). These accessory genes include the regulatory proteins tat, rev and nef, the viral infectivity factor vif, and two proteins of unknown function vpu and vpr. For study of the biological activity of the proteins coded by these genes, site directed mutagenisis or enzymatic digestion followed by religation of the proviral DNA were used to generate viruses with defects or deletions in specified locations in the viral genome. These mutant viruses were transfected into permissive cells and the biological consequences of the specific defects studied in vitro. This approach in our labs (present study) and in that of others has proven to be a very fruitful one for examining viral gene functions and have permitted identification and characterization of the accessory genes of HIV-1.

Sequential expression of the regulatory genes induces modulation of viral expression which appears to be the key event in determining viral replication and latency. Two gene products (tat and rev) have been shown to directly modulate viral expression via transcriptional (tat) and post-transcriptional (tat and rev) mechanisms. The transactivator gene (tat) is critical for virus replication and enhances transcription of genes linked to the long terminal repeat (LTR) (12). The rev gene (formerly known as art and trs) positively regulates the expression of structural proteins but negatively regulates the regulatory proteins including itself (13). In turn, the nef (formerly called 3' orf) gene product is a negative regulator of viral expression (14). The vif (formerly known as sor) gene product is essential for the efficient propagation of extracellular virus in target human cells in vitro. The vpr gene product has not been identified but it appears to be non-essential for viral replication in vitro. Vpu encodes a 16 kd protein and its expression, like vpr, does not appear to be required for viral replication. A replication competent molecular clone of HIV-1 having a

termination codon after the initiating AUG of vpu efficiently produces virus in vitro (15).

A virus very closely related to HIV-1 has been recently isolated from a West African Patient with immunodeficiency (16). While HIV-1 has been associated with the majority of Acquired Immunodeficiency Syndrome (AIDS) cases worldwide, the type-2 virus (HIV-2) has so far been confined primarily to AIDS cases in West Africa (17, 18). Closely related to HIV-2 is the Simian Immunodeficiency Virus (SIV) which has been isolated from captive rhesus macaques with clinical signs of immunodeficiency (19). Interesting parallels can be drawn between the retroviruses HTLV-I, HIV-1, HIV-2 and SIV. Among these are: 1) a common major target cell (CD4+ T-lymphocyte), 2) association with diseases of the immune and central nervous systems, 3) presence of highly related viruses that naturally infect Old World primates, and 4) common regulatory pathways for viral gene expression. Experimental inoculation of SIV in macaques induces immunodeficiency at high frequency, providing an excellent animal model to study pathogenesis, prevention and treatment of human AIDS. Although macaques in the wild are not known to be infected with SIV, several other Old World monkey species (pig tailed macaques, sooty mangabey, macaque menestrina, African green monkeys) appear to be naturally infected with viruses related to SIV_{mac} which is quite divergent from these other simian viruses. SIV_{mac}, however, is closely related to HIV-2, whereas SIV_{agm} is equally distant from both HIV-1 and HIV-2 (20). The biological similarity among these primate viruses suggests evolutionary conservation of their functionally active genes.

The HIV-2 and SIV genomes characterized to date were found to contain open reading frames corresponding to most of the HIV-1 genes, although some differences were observed (21). An additional gene (vpx) was found in HIV-2, SIV_{mac} and SIV_{agm}, and does not have an obvious counterpart in HIV-1. On the other hand, the vpu gene is found only in HIV-1. SIV_{agm} appears to lack the vpr gene which is found in HIV-1, HIV-2 and SIV_{mac}.

Conservation of the amino acid sequence in the putative functional domains of common genes and the biological similarities among these AIDS associated retroviruses would lead one to assume that what applies to HIV-1 should also apply to HIV-2 and SIV. A low level of conservation of the amino acids is observed among the regulatory proteins of SIV_{mac}, SIV_{agm} and the various isolates of HIV-2 (rod, NIH-Z, SBL6669) and HIV-1. On the other hand, a high level of conservation is observed in the amino acid sequence of the gag and pol proteins. A comparison of all regulatory genes indicates that there are some short regions in regulatory genes which are conserved and are involved in

maintaining the structure of the protein. The cysteine/arginine rich region in the first exon of the tat gene and the arginine rich region in the second exon of the rev gene are highly conserved among all these viral isolates (20).

The envelope gene of HIV-1 has been shown to be highly variable among different HIV isolates. Interstrain variations occur consistently within the same areas of the envelope gene. These same regions are also poorly conserved among the HIV-2 and SIV isolates characterized thus far. The HIV-1 transmembrane protein is 41 kD in size, while the SIV and HIV-2 transmembrane proteins are only 32 kD. This difference in protein size is apparently due to the presence of a translation termination codon in the env genes of SIV and HIV-2. Interestingly, the termination codon, is located in the same place in both viruses and is present in most of the SIV strains characterized so far and in an HIV-2 provirus. Several researchers have determined that this premature termination codon was the result of the manner in which the virus was propagated (22). Virus isolated from Hut78 cells, contained the premature termination codon, while virus grown in fresh lymphocytes exhibited full length envelope protein. Results such as these indicate that a better understanding of virus propagation and effects of mutation is required in order to fully understand the pathogenicity of the virus.

C) CURRENT PROGRESS

1) CHARACTERIZATION OF DELETION MUTANTS OF HIV

a) Rationale

To study the molecular basis of infectivity, cytopathogenicity and formation of infectious particles, a series of defective HIV-1 particles were generated with deletions in the non-structural proteins. The consequences of such alterations were examined upon subsequent transfection into recipient cells. The areas of the viral genome selected for closer study included the vif region, the nef and carboxy region of gp4l, and the 5' packaging region (Fig. 1). In the first year, the deletion mutations in these regions were generated, and methods for examining various parameters of viral function in vitro were worked out. In the second year, reported here, these studies were further extended to more fully characterize the biological properties of the mutants and to correlate gene function to specific loci on the viral genome.

Preliminary studies with mutants in the vif region (previously called sor) indicated that this gene is conserved among all HIV isolates and in SIV and HIV-2 (21), suggesting that this gene may be functionally significant. Our initial studies with mutations in this region have shown that vif is required for efficient virus transmission in vitro, and that it acts at a post-transcriptional level (23). Because of its role in enhancing infection, the gene was renamed viral infectivity factor (vif). Further characterization of vif was continued in the second year.

One of the unique characteristics of the HIV-1 transmembrane protein gp41 is that it has an extremely long cytoplasmic domain, which is still undefined. A molecular clone pHXB2D which contains full-length HIV-1 provirus was previously described to produce infectious virions and have cytopathic effects on normal T lymphocytes in vitro. The speculation that the carboxy terminus of the gp41 glycoprotein might have a direct role in T-cell killing by HIV-1 comes from a study in which a variant X10-1, derived from the pHXB2D with the deletion spanning the env and nef genes replicates but does not kill normal T cells (24). We hypothesized that the C-terminus of the gp41 transmembrane protein is vital to viral transmissibility and cytopathicity. We tested this hypothesis by using a panel of mutants which have deletions in the env, the nef, and/or 3' LTR to study the effects of the defined mutations on the replication and cytopathic potential of the virus in the human T-cell lines, H9 and Molt-3.

One approach for the production of defective HIV viral particles which can be used as interfering particles for therapy or a source of vaccine is the production of "empty particles" which do not contain viral RNA, and are therefore incapable of replication. Although much is known about HIV replication and the processing of structural components (gag, pol, and env) in infected cells, the precise mechanism by which genomic viral RNA is preferentially packaged into virion particles is unclear. Such a mechanism, presumably involves a recognition sequence in the viral genome which permits the viral assembly apparatus to specifically select and incorporate its own genomic RNA out of a vast array of viral and cellular mRNA's, and ribosomal and transfer RNA's found in infected cells. Studies in the avian and murine retrovirus systems have suggested that virus particle formation can occur in the absence of genomic RNA (25) and that the sequences which fall between the 5' LTR and gag are critical for virus specific packaging (26, 27, 28). To see whether analogous sequences in HIV are important in selective packaging of genomic RNA, we produced a series of mutants of the biologically active molecular clones pHXB2-D and X10-1 and studied their biological properties.

b) Experimental Methods

Construction of HIV-1 Mutant Clones

A series of mutant clones with changes in discrete portions of the HIV-1 genome were constructed by site directed mutagenisis or by exonuclease cleavage and religation as described in last year's Annual Report. The molecular clone pHXB2Dgpt contains a full length, biologically active HIV-1 genome (9.6 kb) flanked by the long terminal repeat sequences in the plasmid vector pSP62gpt which contains the xanthine guanine phosphoribosyl phosphatase gene. This construct was used for preparation of themutants examined in the present study. Deletion mutations in the carboxy terminal of the envelope gene (nef/gp41) were generated by Xho I digestion of pHXB2, treatment with Bal 31 for varying periods of time, generation of blunt ends with T4 DNA polymerase, and self-ligation with T4 DNA ligase.

Mutations in vif were prepared by site directed mutagenisis to introduce stop codons at various points in the vif frame downstream from the vif/pol overlap (Fig. 1). The 1092 bp Eco RI fragment of the HIV-1 genome was isolated from λBH10 and inserted into an M13 phage vector. Mutations were introduced by use of 25-mer oligonucleotides which introduced stop codons at residue 55 (clone 6.9), 42, (clone 3.3), and 100 (clone 153) (29). The mutated regions were cut out of the double stranded M13 by Eco RI and religated into the corresponding position of pHXB2gpt. The mutant ΔS was prepared directly from pHXB2gpt by digestion with Nde I and Nco I and religation.

Deletion mutations in the 5' region of HIV-1 were prepared from the pHXB2gpt by digestion with BssH II, followed by digestion for various periods of time with Bal 31 exonuclease, and religation. A series of mutations thus produced contained deletions between the LTR and the 5' end of gag. Mutant #3, 11, 25, and Δ293 contained deletions of 53, 59, 10, and 35 nucleotides at positions 224-277, 224-283, 310-320, and 293-328, respectively. In all cases, the splice donor site at position 287 was preserved.

Biological Characterization of Deletion Mutants

Cos-1 cells were transfected with 5 μg of DNA from plasmid mutants, pHXB2D (positive virus control), or pSV2neo (negative virus control). The activity of reverse transcriptase (RT) of the spent medium from the transfected Cos-1 cells was assayed from 2 days to 2 weeks after the transfection by the incorporation of ^{3}H dTTP into TCA

precipitable material. Samples with a ratio of the incorporation using RNA vs DNA templates of greater than 3:1 were scored as positive. Viral transmission to H9 or Molt-3 cells was carried out by cocultivating 5 x 10^5 polybrene-treated H9 or Molt-3 cells with the transfected Cos-1 cells right after the transfection. The coculture was maintained for 48 hours at which time the H9 and Molt-3 cells were separated from the Cos-1 cells. These H9 and Molt-3 cells were then maintained in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS). The production of the gag p24 protein, monitored weekly by immunofluorescent assay of cells fixed in methanol/acetone, was used to demonstrate viral transmission from the transfected Cos-1 to H9 or Molt-3 cells.

Cell-Free Transmission of HIV Mutants

Cos-1 cells were transfected with 5 μg of DNA from each of the mutants, pHXB2D or pSV2neo. After 48 hours incubation, the resulting virus was collected from the supernatant, and clarified of cellular material by centrifugation at 6,000 rpm for 20 minutes. The virus from the clarified supernatant was collected by overnight precipitation with PEG. Serial dilutions of this virus were used to infect H9 and Molt-3 cells. Cells were assayed at weekly intervals for the appearance of cytopathic effect by the exclusion dye 2% trypan blue (TCID50 - Tissue Culture Infective Dose 50%), and expression of HIV-p24 by reactivity with a monoclonal antibody to this antigen using immunofluorescence assays to obtain an inverse geometric mean titer (GMT).

Cytopathogenicity of Cell-Free Virus Preparations of Deletion Mutants

Polybrene-treated H9 cells, 3 x 10^6 in 3 ml RPMI medium containing 20% heat inactivated fetal calf serum, were incubated with 300 x TCID50 of the respective virus preparations (or media as the control) for 1 hour at 37 °C. The cells (in 100 μl medium) were transferred to triplicate wells in 24-well microtiter plates, and 2 ml medium was then added to each well. Plates were fed with 1 ml fresh medium weekly. Cell viability was assessed in 1 and 2 weeks of the incubation by exclusion dye 2% trypan blue.

3. Results

Generation of mutants in nef/gp41

A series of deletion mutants in nef and the carboxy terminus of gp41 were generated about the Xho I site of pHXB2D. The mutants thus generated were grouped into various classes depending on the length of the deletion. These groups included mutants with deletions in nef only; or those which extended into gp41; gp41 plus LTR; or gp41, LTR plus tat/rev. These deletions also resulted in the addition of other amino acids to the end due to a frame shift. The mutants examined in this study and the amino acid changes in env are listed in Table 2.

Cell-to-Cell Transmission Properties of Deletion Mutants in the Carboxy Region of env

Mutants with deletions at the 3' end of the env, nef, and 3'LTR were transfected into cells of the monkey fibroblast cell line Cos-1. Replication was determined by expression of viral reverse transcriptase activity in the cell free supernatants of transfected Cos-1 cells, and by the appearance of HIV-1 p24 in the cells in immunofluorescence assays. The ability of transfected cells to transmit infectious virus to uninfected cells was also examined by culturing transfected Cos-1 cells with polybrene treated cells of the human lymphoblastoid cell lines, H9 or Molt-3 for two days. The H9 and Molt-3 cells were then separated from the Cos-1 cells and incubated separately. Shown in Table 2 is a summary chart reflecting the kinetics of the appearance of Reverse Trancriptase (RT) activity and p24 gag protein expression in H9 and Molt-3 cells. Mutant clones with deletions in the carboxy region of gp41 were observed to be capable of producing reverse transcriptase even when the mutations overlapped into the nef and LTR regions. Mutants LR360 (87-amino acid deletion in the gp41) and LR327 (177-amino acid deletion in the gp41) could not produce this enzyme since their deletions affected the tat/rev genes which are essential for the production of infectious particles. All the other mutants examined could propagate in H9 cells to establish infection in 4 weeks, although two mutants LR295 (14-amino acid deletion in gp41) and LR329 (no deletion in gp 41) propagated slowly at first. The most disparate transmission of mutants in H9 versus Molt-3 cells was observed in mutants LR429 (15-amino acid deletion in the gp41), LR269 (17-amino acid deletion in gp41), LR468 (33-amino acid deletion in gp41), and LR362 (37-amino acid deletion in gp41)

which transmitted relatively efficient'y to the H9 cells but transmitted very poorly, if at all to Molt-3 cells.

Quantitation of Cell-Free Transmission of Mutant Clones in H9 and Molt-3 Cells

To measure the relative differences in the ability of the mutant clones to transmit in a cell-free manner, Cos-1 cells were transfected with the mutant DNA's and the resulting viral particles released to the culture fluid were collected from clarified medium by PEG precipitation. Serial dilutions of this cell-free virus preparation were then assayed on H9 and Molt-3 cells. The dilution which produced a cytopathic effect in 50% of cultures ($TCID_{50}$) was determined. The cells were also examined by immunofluorescence with monoclonal antibody to HIV p24 (BT-3) and the dilution which gave a 50% reduction in the number of fluorescent cells was calculated to obtain an geometric mean titer (GMT). Both the $TCID_{50}$ and GMT results showed excellent agreement (Table 3). Two mutants LR269 (17-amino acid deletion in gp41) and LR362 (37-amino acid deletion in gp41) were highly infectious in H9 cells, having TCID50 values 3158 and I790, respectively, in comparison with the parental pHXB2D virus, which had TCID50 value 2435. In contrast, these two clones were much less infectious in Molt 3 cells, having TCID50 values 558 and 790, respectively.

The kinetics of viral propagation of selected mutants in H9 or Molt-3 cells derived from coculture are shown in Figures 2a and 2b, respectively. Compared to the parental virus, mutants LR295 (14 amino acids deleted from the gp41) and LR329 (33 amino aicds deleted from gp41) propagated slowly but did establish the infection. Mutants LR429 (15 amino acids deleted), LR269 (17 amino aicds deleted), LR468 (33 amino acids deleted) and LR362 (37 amino aicds deleted) could not propagate in Molt-3 cells at all.

In other studies, we analyzed the kinetics of Reverse Transcriptase production and virus replication of selected clones which had deletions involving tat/rev or the 3'LTR region, rendering them incapable of growth after transfection into Cos-1 cells. Table 4 shows the results of experiments involving detection of p24 (gag) production after coculture with susceptible H9 and Molt-3 cells. Only LR204 and LR318 (-7+0 and 6+0, respectively) appear capable of appreciable replication in H9 cells. None of these clones were capable of establishing infection in Molt-3 cells.

Determination of Cytopathic Effects of Mutant Clones

Viral particles of selected mutants at 300 times their TCID50 concentrations were used to infect H9 cells and cell viability was monitored weekly for a period of two weeks. The cell viability, used as the measurement of viral cytopathogenicity on H9 cells, showed that in two weeks, mutants X10-1 (5-amino acid deletion), LR295 (14-amino acid deletion) and LR269 (17-amino acid deletion) had little or no cytopathic effect by two weeks (Fig. 3). When the deletions extended to 33 amino acids (LR468), cell viability decreased to 39%. As deletion further extended to 37 amino acids from the 3' end of the gp41 (LR362) cell viability decreased to 32%. This reduction in viability approached that obtained with the parental viral clone pHXB2D (24% viability). It is interesting to observe that mutant X9-3 (5-amino acid deletion) had 41% cell viability.

The cytopathic potential of the different C-terminal deletion mutants was also examined using a new technique involving coculture of infected cells with exquisitely susceptible target cells. The cell lines ATH8 and HPB-ALL have been shown to be particularly susceptible to rapid killing by HIV, even at relatively low doses of virus. The ability of mutant virus produced in H9 or Molt-3 cells to kill these indicator cell lines by a cell-associated mechanism was examined . Unexpectedly, mutants previously described as non-cytopathic (X10-1 and X9-3) as cell-free virions appeared to show marked cytopathic effects when cocultured with the indicator cells ATH8 and HPB-ALL (Table 5). Mutants LR495 and LR362 which transmitted poorly to Molt-3 cells, nevertheless were still capable of killing the indicator cells. The indicator cell lines were more susceptible to killing by X9-3 when it was propagated in Molt-3 cells than when the same mutant was propagated in H9 cells. The cytopatogenicity of LR295, in contrast, was greater when this mutant was propagated in H9 cells. These observations suggest that cellular factors also contribute to the cell killing property of HIV. These cellular factors interact with different viral determinants in bringing about the cytopathic effect.

Expression of gp41 by the deletion mutants in the carboxy region of env

The production of the various viral proteins by the mutants in the carboxy region of env was examined using standard radioimmunoprecipitation techniques with ^{35}S-methionine labeled infected H9 cells and sera from infected patients (see Experimental Methods in Section 3). All the major viral proteins, including p24, gp41, gp65, gp120

and gp160 were clearly produced in response to infection with these mutants (Fig. 4). While there is a suggestion of minor differences in gp160, there is no discernible difference in the migration of gp41 bands. Radioimmunoprecipitation of mutants with deletions large enough to be clearly seen by this technique would not be possible if these clones were not capable of replication in neoplastic lymphoid cell lines.

Neutralization of Cell-to-Cell Transmission of HIV-1

In the first year of this contract, we had shown that mutations in vif (sor) are not required for HIV virion formation, but influence viral transmission in vitro. The mechanism by which these mutants affect cell-to cell transmission was further evaluated during the second year. Using soft-agar cloning techniques, we have developed a number of cell lines expressing different vif mutant viruses. Previous studies have shown cell free transmission of these mutants to be defective (23). Since some of these lines are up to 95% positive for gag expression, they are very useful as inocula for neutralization studies for fresh polybrene treated Molt-3 cells. A standardized system using fixed numbers of Mitomycin-C treated Molt-3 cells infected with the various vif deletion mutant clones (ΔS, 3.3, 6.9, 153) was developed as an inoculum for fresh polybrene treated uninfected cells. This system shows predictable replication characteristics which allows investigation of the molecular basis of cell-associated transmission of HIV, by determining the effects of antibodies directed against specific cellular and viral structures. For example, antibodies such as OKT4A, OKT4, and Leu3a against CD4, Leu10 against HLA-DQ, Anti-HLA Class II antibodies, anti-gp120, gp41 and p24 monoclonals, and selected infected patient sera can be used to examine the role of various antigens in cell-to-cell transmission. Shown in Table 6 are typical kinetics for this assay. Up to 30-50% of the cells express HIV-p24 at 7 days post infection, and infected cells continue to persist in the culture for up to 28 days. Various vif mutant infected cells lines have been cloned in order to obtain more homogeneous populations to use as inocula.

In an attempt to dissect the molecular basis of cell-free or cell-associated transmission , we examined the ability of a variety of human HIV sera to neutralize the transmission of the mutant viruses to H9 cells. For this assay, H9 cells were infected with 4 times the infectious dose (4 x TCID50) of HXB2D or HTLVIIIB (wild type virus) in the presence of various dilutions of sera. Cell-associated transmission was determined in a similar manner, except that the cell lines expressing the vif mutant viruses were used as the source of the infecting virus. Expression of virus was

monitored at 2 weeks post infection by immunofluorescence with anti HIV-1 p24. The serum dilution which gave a 50% reduction in titer was determined. OKT4A, but not OKT4 is extremely efficient in blocking cell-associated transmission, implying that the gp120-CD4 interaction is still required for cell-to-cell spread of the mutant viruses. The results with the human sera are shown in Table 7. Some sera (HS06 and HS12) neutralize cell-free transmission but not cell-associated transmission of the mutant virus. Other sera (HS02 and HS22) neutralize cell associated, but not cell-free transmission. These results suggest that different epitopes may be involved in the cell-cell versus cell-free transmission of HIV, and these differences will be further characterized in the third year.

Characterization of Packaging Defective Mutants

Several candidate packaging mutants were examined for their ability to give rise to infectious (RNA containing) virus particles. Plasmid DNA from each clone was transfected into Cos-1, and the culture supernatants of transfected cells were analyzed for the presence of reverse transcriptase activity and the appearance of virus particles by electron microscopy. These supernatants were also examined for the presence of infectious particles by cocultivation with recipient H9 cells. The transmissibility of the mutant viruses was monitored by assessing the increase in HIV expressing cells in the coculture H9 population with time.

Clones #3, #11, #79, and Δ293, all gave rise to morphologically normal virus particles, as examined by electronmicroscopy, and were capable of eliciting Reverse Transcriptase activity upon transfection of Cos-1 cells. The transmissibility of viruses from clones #11, #3, and #79, however, was impaired with respect to wild type virus. These viruses were poorly infectious while virus derived from clone Δ293 appeared to be resistant to propagation (Figure 5). This data indicates, that although viral particles and virus specific enzymatic activity is produced at near wild type levels, the infectivity of these mutant viruses is impaired.

Viral RNA Production by Packaging Mutants

The transfected cells and the resultant viruses were examined for evidence of viral RNA production and packaging. Cos-1 cells were transfected with plasmid DNA from clones #11, Δ293, and pHXB2 (wild type virus). RNA was prepared using the hot-phenol method from cellular lysates, and analyzed by Northern blotting.

Transcripts of sizes 9.5 kb, 4.5 kb, and 2.0 kb were evident in all transfected cells, indicating that viral RNA expression by these mutants was not compromised (data not shown).

The relative amounts of viral RNA produced by these mutants was also analyzed on slot blots. Virus was collected from the supernatants of transfected cells, clarified, and concentrated by ultracentrifugation. The virion RNA was extracted, dot-blotted on nitrocellulose membranes and hybridized with ^{32}P pHXB2 DNA. The results indicate that virus derived from Δ293 and #79 contain unusually low levels of HIV specific RNA relative to that obtained from wild type virus or mutants #3 and 11. Further analysis will be needed to determine whether the reduction in the amount of viral RNA in the cell free virus in Δ293 and #79 represent an impairment in the ability of these clones to efficiently recognize and package genomic RNA.

d) Discussion

Studies of the biological activity of mutations in the 3' env/nef region have shown that mutants of up to 37 amino acids deleted from the 3' end of the gp41 are still capable of producing reverse transcriptase upon transfection into Cos-1 cells and of transmitting virus to H9 cells. However, mutants with 15 or greater amino acid deletions cannot propagate in Molt-3 cells. This preferential transmission in H9 cells suggest that certain cellular factor(s) of Molt-3 and the area between the last 14 to 15 amino acids of the gp41 seem to work in an accord to modulate viral transmissibility and that cellular tropism might play a role in viral transmission. Transmission of mutants LR429, LR269, LR362, LR468 was markedly reduced in Molt-3 cells compared to H9 after coculture with transfected Cos-1 cells. Two of these four clones, LR468 and LR429 exhibited poor cell-free infectivity for Molt-3 cells, but still showed preferential transmission in H9 cells, indicating that the cell-free route alone was not sufficient to establish a successful infection. Therefore, the areas between the last 15 and 17, and between the last 33 and 37 amino acids of the gp41 appear to be important to cell-to-cell transmission. In addition, the cytopathic effects of mutant clones with C-terminal deletions of only 5 amino acids (mutants X10-1 and X9-3), have previously been shown to be reduced, and the present study demonstrates that X10-1 is much less cytopathic than X9-3.

These results definitively localize the critical region for cell-cell transmission to the 17 carboxy-most amino acids of gp41. Two functional areas of importance in this very small region can be postulated. An area important in cell killing lies in the last 6

amino acid residues, or perhaps in only the last 3 amino acid residues since X10-1 differs only in the highly hydrophobic 'ILL' sequence in this region. The other area, important for cell-to-cell transmission lies in the last I7 amino-acid residues, but does not include deletion of the last cysteine residue in gp41. The use of the highly sensitive cell lines ATH8 and HPB-ALL have permitted us to more closely examine the cell killing properties of the mutant viruses. Studies with these cells have shown that even when transmission to Molt-3 cells can not be demonstrated by some of these mutants (LR429 - LR362), they are still capable of killing the indicator cell.

The studies on the cytopathicity of the C-env mutants suggest that the region between the last 14 and 17 amino acids seems to be important to viral cytopathicity since the cytopathicity started to be reduced as deletions extended beyond this region. We speculate that deletion of 14 amino acids from the 3' end of the gp41 altered the conformation of this protein in such a way that the virus became less cytopathic. When the deletion went further to 33 amino acids and beyond, this effect was reversed due to another alteration of the conformation. Perhaps the most significant observation is that the cytopathic effect of the C-terminal gp41 deletion mutants in coculture may far exceed that of cell-free virions, and equal that of wild-type virus. While this is somewhat discouraging, it certainly indicates that any supposedly attenuated strain of HIV will need to be tested in this fashion (cell-associated cytopathogenicity). These studies therefore suggest that caution should be used in selecting candidate immunogens for vaccination derived from the HIV env region, since they might themselves be cytopathic for susceptible cells. Furthermore, novel therapeutic approaches such as those being developed to specifically block tat functions may be ineffective unless they are designed to completely prevent expression of HIV in infected cells.

The mechanism of cell to cell transmission by mutants in vif has been studied with the aid of specific antisera to cell and viral determinants. These studies can help to dissect the viral and cellular components required for efficient cell-to-cell transmission. The differential effects of various human sera to block either the cell-free or cell-associated transmission route suggest that different epitopes are involved in these modes of transmission. These studies will be further extended in the next year.

The work with the packaging deletion mutants have identified two mutants, Δ293 and #79 as potential candidates for production of empty particles. These mutants synthesize all classes of viral mRNA, but the decrease in infectivity and viral RNA content of virions suggest a possible defect in packaging. These mutants will be more carefully analyzed in the next year.

2) STUDIES OF HIV-1 TAT FUNCTION

a) Rationale

The transactivator gene, tat, is an extremely strong positive regulator of the expression of HIV genes, enhancing the production of all viral structural proteins, including itself (12). Since this gene may play an important role in reactivating latent virus, and therefore in the progression to disease, a clear understanding of the mechanism of tat activation is necessary to design regimens to keep the infection under control. The tat gene product has been shown to enhance transcription of genes linked to the long terminal repeat (LTR) of HIV. In the first year of the contract, we have identified the enhancer element, an Sp-1 binding site, and the tat response region (TAR) within the HIV-1 LTR. To further study the regulation of genes linked to LTR, we set out to examine other viral and cellular factors/proteins which play a role in HIV activation by tat. Our studies had suggested that tat functions at both the transcriptional level by promoting increased transcription of viral mRNA, as well as at the post-transcriptional level. The mechanism of postranscriptional activation by tat, however, was not well defined and required further study. In the second year, an in vitro system for examining the post-transcriptional effects of tat was designed, which would allow us to study these mechanisms in more detail.

The enhancement of HIV transcription by tat proceeds by a complex mechanism that involves the interactions between tat protein, the binding elements in the LTR, and various other cellular or viral proteins or factors. Several reports have described the activation of HIV replication and production of virus from latently infected cells by agents that induce lymphokine synthesis and secretion (30, 31). Jurkat cells have been used extensively in studies of the production of interleukins in response to various external stimuli. By treating Jurkat cells with a combination of phytohemagglutinin (PHA) and phorbol myristate acetate (PMA, 4-b-phorbol-12-b-myristate-na-acetate), or either agent individually, transient expression of a reporter gene linked to the HIV-LTR was shown to be elevated compared to similarly transfected Jurkat cells grown in normal media (32, 33). This response was amplified if the HIV trans-activator gene (tat-III) was cotransfected with the HIV-LTR reporter gene construct (13). Induced expression of the reporter gene was shown to coincide with the appearance in nuclear extracts of an activity that retards the migration of a DNA fragment containing the HIV enhancer sequence in a gel retardation assay (33). This activity was proposed to be identical to the NF-KB (34, 35) factor that is detected in

kappa-immunoglobulin light chain producing cell lines by the gel-retardation assay. In the present studies, we wished to further investigate the role of cellular proteins in the regulation of genes linked to the HIV-LTR, and to examine the relationship between the LTR binding proteins in stimulated Jurkat cells to those found in H9 cells.

b) Experimental Methods

Preparation of Biotinylated Oligonucleotides.

Typically 2 nmoles of single-strand oligonucleotide were biotinylated in water, and then annealed to its complementary strand. Photoprobebiotin (1 µg/µl-Vector Labs) was added to the nucleic acid solution in an amount equal to the mass of nucleic acid. The tube was placed in an ice water bath so that the surface of the mixture was 7 cm below the sunlamp, a glass microscope slide was placed over the mouth of the tube, and the mixture was exposed to the sunlamp for 15 minutes. All procedures prior to exposing the reaction to the sunlamp were conducted in a darkened room. The biotinylated strand was annealed to its complementary strand by adding the complementary strand and 1/10th volume of annealing solution. The mixture was placed at 80 °C for 2 minutes and then at 37 °C for 1 hour. The mixture was adjusted to a pH >8.5 by the addition of 1 M TrisHCl, pH 9.0, and extracted three times with equal volumes of n-butanol. The volume was adjusted to 10 pmole/µl nucleic acid, and then stored at -20 °C.

Preparation of Nuclear Extracts.

A typical assay contained the proteins extracted from the nuclei of 1 x 10^8 cells. Lymphoblasts used in this study were maintained at 5 x 10^5 to 1 x 10^6 cells/ml in RPMI 1640 + 15% FCS. Six x 10^8 cells were labeled in 1.0 ml DMEM-methionine containing 1.5 mCi; ^{35}S-methionine at 37 °C for 30 minutes in a 35 mm dish. After labeling, the cells were pelleted by a 30 second spin at 500 x g, washed two times with phosphate-buffered saline (pH 7.3), washed one time in 30 mM Tris pH 8.0, 1 mM KCl and suspended in 1.0 ml of this solution. The cells were disrupted by 10-20 strokes in a 1.0 ml dounce homogenizer (Type B). The crude nuclei were pelleted by a 15 second spin at 1000 x g and suspended in a solution containing 0.1% NP40, 1.5 mM $MgCl_2$ and 420 mM KCl (1.4 ml per 3 x 10^8 harvested cells). A final concentration of 300 mM KCl was sufficient for extraction of the oligonucleotides. The extraction was carried out at 4 °C on a slowly rotating wheel for 1.5 hours. The extract was then centrifuged for 5

minutes at 13,000 x g at 4 °C. The supernatant was removed and the pellet rinsed with 1.0 ml of the above solution and recentrifuged. The supernatant was combined with the original volume of extract and the KCl was adjusted to a final concentration of 100 mM. The extract was preadsorbed with streptavidin-agarose, by adding 1.2 ml of extract to a 75 µl pellet of agarose beads. Preadsorption was for 20 minutes at room temperature. The mixture was centrifuged at 13,000 x g for 5 minutes at room temperature. The supernatant was carefully removed so as to leave 20 µl of supernatant over the pelleted beads.

Binding Assay.

The preadsorbed nuclear extract was placed in eppendorf tubes at a volume of 1.0 ml. When competitor nucleic acid was used, it was added first and allowed to mix with the extract for 15 minutes at room temperature. The competitor was poly(dI-dC)-(dI-dC) (Pharmacia, Piscataway, NJ), and was added at 40 times the concentration of the double stranded oligonucleotide. The amount of oligonucleotide added was 100 pmoles/ml of mix unless otherwise noted. After 20 minutes on a slowly rotating wheel, at room temperature, the mix was centrifuged for 2 minutes at 13,000 x g. All but 20 µl of the mix was removed and added to 35 µl pellet of streptavidin-agarose. After rotating for 30 minutes at room temperature, the mix was centrifuged for 15 second at 13,000 x g. The supernatant was removed and the beads washed three times with a solution containing 100 mM KCl and 1.5 mM $MgCl_2$. The bound proteins were analyzed by polyacrylamide gel electrophoresis. Immediately prior to loading the gel, the beads were thawed, placed in a boiling water bath for 3 minutes, centrifuged for 2 minutes at 13,000 x g and the supernatant loaded onto the gel.

c) Results

Characterization of cellular proteins that recognize the HIV enhancer

Nucleic acid affinity procedures were designed to identify cellular proteins which bind the enhancer element of HIV and may be involved in directing expression of genes linked to this region. For this purpose, we developed a DNA-affinity precipitation assay analogous to immunoprecipitation studies of cellular proteins. The principle is to separate specific DNA binding proteins from complex mixtures of proteins, and then to resolve them by gel electrophoresis. Synthetic oligonucleotides or restriction fragments

were covalently modified by the addition of a biotin group and mixed with either whole cell or nuclear protein extracts. The protein-nucleic acid complexes were collected on streptavidin-agarose beads. The proteins were eluted from the beads and resolved by standard gel electrophoresis techniques. A routine assay can be accomplished within 3 hours, excluding gel running time. The assay can therefore be used to directly characterize proteins from individual cell lines that recognize a particular nucleic acid sequence and to compare such proteins to those that bind to other regulatory elements. This assay can also be used to demonstrate cell specific or response specific binding proteins by analyzing different cell lines under various growth conditions. In addition to isolating those proteins that interact with a nucleic acid-protein complex, these assays can be used to identify proteins that bind specifically to the nucleic acid. This simple assay is meant to complement current, widely used procedures such as gel retardation and nuclease protection assays of protein-nucleic acid interactions.

Deletion analyses of the HIV-LTR during the first year have revealed an enhancer element between -105 and -80 base pairs upstream of the transcription start site and both positive and negative control regions at sites upstream of this enhancer element. In the present studies we examined the interactions of cellular proteins with the genetically well characterized HIV enhancer. This enhancer (-104 to -80) contains a 100 bp direct repeat, GGGACTTTCC, that is 100% homologous to enhancers to two DNA viruses, SV40 (36) and human cytomegalovirus (37), and to a sequence in the kappa-immunoglobulin light chain gene enhancer region (33). For use as probes we synthesized oligonucleotides containing two complete, tandem copies of the region extending from -106 to -79 (Fig. 6) which we refer to as HIVEN3c 1 and 2. Parallel mutant oligonucleotides called HIVEN3m 3 and 4 contain three point mutations within each decamer repeat. The mutant construct was chosen because it had previously been shown to abrogate PHA $\pm$ tat III inducible activity of HIV-LTR reporter gene constructs (33). Fig. 6, lane 4, shows the pattern of proteins that bind to the wild-type annealed oligonucleotides HIVEN3c1/2 using nuclear extracts prepared from H9 cells. The pattern is complex with proteins of 130 kD, 110 kD, 86 kD, 40 kD, 35 kD, and 28 kD in size being easily detected. These proteins are not detected in control reactions lacking biotinylated oligonucleotides (Fig. 6, lane 3). Using mutant probe HIVEN3m3/4 there is a significant decrease in binding of the protein(s) migrating at 86 kD (Fig. 6, lane 5); whereas the remaining proteins are still precipitated. Comparison of the protein patterns with these two DNA fragments define four classes of proteins: 1.) non-specific proteins such as actin (42 kD) that are precipitated by the streptavidin-agarose beads; 2.) proteins enriched by binding the wild-type fragment and the mutant fragment, such

as 130 kD and 110 kD; 3.) a protein(s) preferentially associated with the wild type fragment, 186 kD; and 4.) a protein(s) designated by the "*" that bind to the mutant oligonucleotide probe.

The relationship between the LTR binding proteins in stimulated Jurkat cells to those found in H9 cells was compared. Two human B-lymphoblast cell lines expressing exclusively either the kappa- or the lambda-immunoglobulin light chains (38) were also included. The kappa-immunoglobulin producing cell line provides a putative NF-KB positive control for comparison with the PHA-induced Jurkat cells, and the lambda-immunoglobulin producing cell line was chosen to determine if they possess similar and/or unique proteins.

To address this issue we carried out a series of gel-retardation assays (25-27) using a cloned HIV enhancer 12-base pair probe (-GGGACTTTCCAG-) in addition to the microscale affinity assay. Fig. 6 shows that protein extracts from each of the cell lines assayed contained activities that bind to this sequence element. These patterns are more complex than previously reported (39). The specificities of these interactions were determined by the addition to the retardation reactions of competitor oligonucleotides that were the same biotinylated probes used in the binding assays. Band 1 is found only in H9 cells, not in the B-lymphoblast cell lines nor in the Jurkat cells $\pm$ PHA. It is competed specifically by the HIVEN3c1/2. Band 3 (a poorly resolved doublet) is found in H9 cells, in both kappa- and lambda- light chain producing B-lymphoblast lines, and is substantially induced in Jurkat cells upon stimulation with PHA for times previously shown to be sufficient to induce transcription of HIV-LTR linked reporter genes. This behavior correlates with the activity referred to as NF-KB in that lectin stimulated Jurkat cells and kappa-immunoglobulin producing B-lymphoblasts both form the band 3 complex. The lambda-immunoglobulin producing cells also appear to have an identical activity (Fig. 6, BN10, band 3). These assays show that an 86 kD protein (Band 3, designated HIVEN86A protein) is present in H9 cells and both B-lymphoblast cell lines. In Jurkat cells, stimulation with PHA results in a significant increase of this protein binding to the HIVEN3c1/2. These results, therefore, have identified a protein of 86 kD which binds to the HIV enhancer that appears following stimulation of Jurkat cells with PHA.

Studies on the Post-transcriptional Effects of tat

The post-transcriptional effects of tat were examined using a system in which any transcriptional control by tat, is eliminated by looking just at post-transcription

events. Plasmids were constructed which contained the T7 RNA polymerase recognition sequences upstream of the TAR region joined to the chloramphenicol acetyl transferase gene in plasmid L164.2 as shown in Figure 7. This construct was digested with Bam HI and capped RNA transcripts were synthesized in vitro using T7 RNA polymerase (Fig. 8). The resulting transcripts contained bases 1 to 80 of the HIV mRNA linked to the CAT gene as determined by Northern analysis and by primer extension. These RNA's were then microinjected into Xenopus oocytes in the presence and absence of tat expression vectors. Extracts of these oocytes were prepared and analyzed for CAT activity. Co-injection of the HIV TAR-CAT RNA with tat expression vectors resulted in a 30-fold increase in the amount of CAT synthesized relative to that with HIV TAR-CAT RNA clone. This is direct evidence that the transactivating events mediated by tat III are at least in part post-transcriptional. We are presently generating a number of mutant TAR messages by specific deletions and substitutions made in the original plasmid used to generate the message, L164-2. The mutant message important in the post-transcriptional events surrounding tat activation.

Binding of Cellular Proteins to TAR

One approach for determining the contribution of post-transcriptional events in the transactivating action of tat III has been to determine specific cellular proteins that bind to the tat responsive region known as TAR. To do this we have generated and purified an 80 base RNA sequence that represents the isolated TAR region. This has been accomplished by isolating a 2.8 kb region from the plasmid L164-2 that codes for the TAR region and is downstream from the T7 promotor. This gel-purified region was then used in an in vitro transcription reaction with the T7 polymerase along with the appropriate nucleotides to generate the 80 base message. The product was phenol extracted, ethanol precipitated and biotinylated. It was then run in an acrylamide gel which confirmed the size of the fragment. The product has also been capped using m7Gppp5G in the original reaction. This purified RNA will be used in whole cell protein binding assays as well as in gel shift assays to determine what important cellular proteins might be involved in post-transcriptional activities mediated by tat.

d) Discussion

As was demonstrated for H9 cells, the use of the binding assay can lead to the identification of specific nucleic acid binding proteins in gel patterns derived from

separation of whole cell extracts. The identification and resolution of the specific HIV enhancer binding proteins on polyacrylamide gels permits the direct determination of the effect any specified manipulation of the cell has on the behavior of each polypeptide. General application of this approach to the study of defined nucleic acid control sequences has the potential of providing substantial biological information both before and after the protein is purified. This report represents the initial step in the systematic application of the binding assay to define inducible and cell type-specific cellular proteins that interact with nucleic acid motifs determined to be of regulatory significance.

The detection of the inducible HIVEN86A polypeptides in PHA-stimulated Jurkat cells may indicate that they are involved in the enhancement of HIV-LTR mediated transcription previously reported (32, 33). The fact that the same protein is detected in a kappa-immunoglobulin producing cell line suggests HIVEN86A is a candidate for NF-KB activity (32, 33, 34). The demonstration that H9 cells and lambda-immunoglobulin producing cells have the same gel-retardation activity as stimulated Jurkat cells and kappa-immunoglobulin cells extends the cell type and differentiation-stage previously reported for the constitutive expression of NF-KB activity (32, 33, 34). It may be that HIVEN86A is either completely or partially responsible for NF-KB activity. Purification of these polypeptides is the first step in elucidating the biochemical properties of each HIV enhancer binding polypeptide. The binding assay will provide a very convenient method for monitoring such purification efforts.

The availability of the TAR transcripts wihich have been generated during this year will permit more defined examination of the post-transcriptional effects of tat. The microinjection experiments using transcripts of TAR linked to the CAT gene have demonstrated that such effects can account for a 30-fold increase in the amount of CAT synthesized. The experiments with the protein binding assays with the in vitro synthesized TAR transcripts will also be very useful in examining the role of cellular proteins in the post-transcriptional activities mediated by tat.

3) MOLECULAR STUDIES OF THE HIV-2 AND SIV GENOMES

a) Rationale

A genetic comparison of HIV-2 and SIV_{mac} to HIV-1 revealed that all three retroviruses share common biological features and genetic structure. Equivalent open reading frames corresponding to most of the HIV-1 genes were observed in HIV-2 and SIV_{mac}, along with a few differences (Fig. 9). An additional gene (vpx) was found in HIV-2, SIV_{mac} and SIV_{agm} which has no obvious counterpart in HIV-1. To establish whether the vpx is a gene, we studied its expression in HIV-2 infected individuals and in infected cells in vitro. A HIV-2 proviral DNA fragment containing this gene was expressed in E.coli, and the recombinant protein was used in an immunoblot assay. The vpx protein was recognized by the sera of HIV-2 infected people but not by the sera of SIV_{mac} infected monkeys and HIV-1 infected humans. A rabbit antiserum against the recombinant vpx gene recognized a 16 kD protein in HIV-2 infected cells. The vpx protein was found not to be glycosylated or phosphorylated, was localized in the cytoplasm of HIV-2 infected cells, and was found in the mature virion.

A second feature that differentiates SIV_{mac} and HIV-2 from HIV-1 was the smaller size of the transmembrane portion of the envelope protein. The size of the transmembrane protein is 32 kD in SIV_{mac} and HIV-2 compared to 41 kD in HIV-1. The presence of this truncated form of the transmembrane glycoprotein in SIV_{mac} and HIV-2 virions is related to the presence of a premature translation termination codon in the env gene. Since the carboxy terminus of the envelope transmembrane protein has been implicated in the cytopathic effect of HIV-1, we decided to investigate the expression of this region and to correlate this expression with pathogencity in vivo. Two synthetic peptides were generated from the inferred amino acid sequence of SIV_{mac}. Their reactivity were tested by Western blot against the sera of infected monkeys as well as against sera of HIV-2 infected humans. The protein synthesized from this open reading frame is expressed in vivo. However, no correlation was found to exist between its expression and disease progression.

In continuation of our studies of the HIV genomes, the functions of the accessory genes in HIV-1/HIV-2 are being investigated in vivo in order to establish an animal model for vaccine testing. A biologically active clone of HIV-2 has been obtained, subjected to DNA sequence determination and immunological studies. The infectivity of the resulting cloned virus is under investigation along with characterization of virus

recovered from rhesus macaques. These studies should shed some light on changes which occur in vivo during infection.

b) Experimental Methods

Virus Propagation

SIV_{mac}, HIV-$2_{SBL6669}$, and HIV-2_{NIH} were grown in the human T cell line Hut78 and the same stock of concentrated virus was used to inoculate macaques and baboons. Viral production was determined by reverse transcriptase (RT) activity assay of infected cells.

Peptides PT-1 and PT-2 and rabbit antisera

The peptides used in this study were obtained from Cambridge Biochemical Corporation and were synthesized using solid-phase peptide synthesis technology. Rabbit antisera were obtained from rabbits immunized four times with peptide PT-1 coupled to keyhole limpet hemocyanin (KLH).

Immunoblot assay

For detection of antipeptide immune reactivity, human and monkey sera were diluted 1/100 and used against 1 µg of each peptide spotted onto nitrocellulose filters. Bound antibodies were detected using ^{125}I-labeled Staphylococcus aureus protein A at 5 x 10^5 cpm/ml 5% dry milk. For Western blot assay, 1 x 10^7 cells infected with HIV-1, HIV-2, or SIV_{mac} or virions were lysed in 500 µl of radioimmunoprecipitation assay (RIPA) buffer (40) and 50 µl were run on an SDS-PAGE gel (12.5%)(41). After electrophoretic transfer (60 V overnight) to nitrocellulose filters, the samples were reacted with the rabbit anti-PT-1 sera (1/50 diluted) and stained with ^{125}I-Staphylococcus aureus protein A. The molecular weights of reactive bands were calculated relative to the migration of the Amersham "rainbow" markers.

Immunoprecipitation of Viral Proteins

Cells infected with HIV-$2_{SBL/ISY}$ or SIV were incubated in medium supplemented with [^{35}S]-methionine and [^{35}S]-cysteine (100 µCi/ml; 1 Ci = 37

GBq) for 4 hours and pelleted. The supernatant was centrifuged at 20,000 rpm for 1 hour to pellet the labeled virus. The labeled viral lysate was precleared overnight with normal human sera and Sepharose-bound protein A. Aliquots of the precleared cell lysate were incubated with sera from HIV-2 infected humans or SIV infected monkeys and the immunocomplexes were isolated with Staphylococcus aureus protein A bound to Sepharose. The samples were electrophoresed on a 10% SDS/polyacrylamide gel and the gel was treated with enhancer for 30 minutes, dried, and autoradiographed.

Transfection in Neoplastic T Cells

Ten million Hut78 cells were used for each transfection. Forty million cells were resuspended in 40 ml of RPMI 1640 medium with 10% fetal calf serum (FCS)(GIBCO), and incubated at 37 °C for 5 hours. After incubation, the cells were washed with RPMI 1640 medium without FCS and aliquoted (10 million cells) into four tubes. The cells were resuspended in 4 ml of RPMI 1640 medium without FCS containing 50 mM Tris-HCl (pH 7.4) plus 10 µg of DNA. Subsequently, 1 ml of 5 x DEAE dextran solution (25 mg/ml) in RPMI 1640 medium without FCS in 1 M Tris·HC1 (pH 7.4) was added to each tube. The samples were incubated at 37 °C for 1 hour with gentle shaking. After incubation, the cells were pelleted at 1500 rpm and washed twice at room temperature with complete medium (RPMI 1640 medium with 10% FCS). The following day, 10 ml of fresh medium was added. Viral production was monitored by testing for magnesium-dependent reverse transcriptase (RT) one week post-transfection. For the RT assay, the proteins contained in the supernatant were precipitated with 30% PEG/0.4 M NaCl and the pellets were resuspended in VSB (42). Reverse transcriptase activity was determined by precipitable counts of incorporated ^{3}H-thymidine.

Immunofluorescence on the Infected Cells

Immunofluorescent staining of infected cells was performed with serum from individuals infected with HIV-2. The cells were pelleted, fixed with 50% methanol/50% acetone for 10 minutes, and incubated with 15 µl of human serum diluted 1:40 with phosphate-buffered saline (PBS) for 30 minutes at room temperature. The slides were washed with PBS and incubated with Molt-3 conjugated anti-human antibodies in the dark for 30 minutes. Positive cells were scored under a UV light microscope. Electron microscopy on the infected cells was performed as described by Biberfeld et al (43).

Infection of Target Cell Lines

Concentrated virus was obtained from the transfected cell line Hut78 constitutively producing the HIV-$2_{SBL/ISY}$ virus. The equivalent of 1000 $TCID_{50}$ (tissue culture 50% infective dose) infecting virus was used to infect the cell lines H9, Molt-3, U937, Hut78, CEM, MT-2, and the T-cell clone 55. Cells (5×10^6) from each culture were treated with Polybrene (Sigma) at 5 µg/ml for 1 hour. The cells were washed with 1 x PBS and incubated for 1 hour with the virus. At the end of the incubation period, the cells were washed again and resuspended in RPMI medium-10% FCS. Immunofluorescence and RT activity were measured every 3 days as described above. Cell viability was calculated by dividing the number of cells not incorporating trypan blue color by the total number of cells. The number of syncytia was counted under a light microscope.

Western Blot Analysis

Cells from infected cultures were pelleted and the supernatants were centrifuged at 20,000 rpm for 1 hour to pellet the virus. The viral pellet was resuspended in 1 x RIPA buffer (5 mM phenylmethylsulfonyl fluoride,75 mM NaCl, 25 mM Tris·HCl pH 7.5, 0.5% SDS, 5% Triton X-100, 5% deoxycholic acid). The lysed virions were loaded on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters. The filters were treated with 5% dry milk in PBS for 1 hour in order to block non-specific binding sites (40). The antibodies used are designated in the figure legends. Iodinated Staphylococcus aureus protein A was used to detect immunocomplexes.

Molecular Cloning and Sequencing of Proviral DNA

A λ phage library was constructed from the genomic DNA isolated from Hut78 cells infected with the HIV-$2_{SBL6669}$ isolate. The DNA which was partially digested with Sau 3A, was fractionated on a linear 10-40% sucrose gradient and the 20 kb fraction was ligated to EMBL-3 arms (44). The ligated DNA was packaged in vitro using the Stratagene gigapack. Recombinant clones were obtained from the library using the SIV gag (B16) and envelope (SS35) probes (45). A recombinant clone, designated HIV-$2_{SBL/ISY}$ was purified. Several subclones of the insert DNA were generated in the Bluescript vector (44). The nucleotide sequence of HIV-$2_{SBL/ISY}$ was determined by

the Sanger dideoxy chain termination method using Sequenase and the chemical modification/cleavage method of Maxam and Gilbert (46, 47).

Southern Blot Analysis of HIV-2 Infected Cells

The total cellular DNA from cell lines infected with HIV-$2_{SBL6669}$, HIV-2_{NIHZ} and HIV-$2_{SBL/ISY}$ was digested with Bam HI, Xba I, and Eco RI and electrophoresed on a 0.8% agarose gel. As a further control, the DNA of the SIV_{mac} infected cells was cleaved with the same enzymes. The gel was denatured, neutralized, blotted to nitrocellulose filters as described (48) and the filters were hybridized to the labeled probes B16 or SS35 (45).

c) Results

Studies of the vpx Region

In continuing our analysis of the comparison of the HIV-1 and HIV-2 genomes, we have focused attention on a portion of the HIV-2 genome containing an open reading frame (designated vpx) which does not have a counterpart in HIV-1. To establish whether vpx is a gene, we studied its expression in HIV-2 infected individuals and in infected cells in vitro. An HIV-2 proviral DNA fragment containing the vpx was expressed in E. coli and the recombinant protein was used in an immunoblot assay. The vpx protein was recognized specifically by the sera of HIV-2 infected people but not by the sera of SIV infected monkeys or HIV-1 infected humans. A rabbit antiserum raised against the recombinant vpx protein recognized a 16 Kd protein in HIV-2 infected cells (Fig. 10).

Molecular analysis of the native vpx protein revealed that the protein was not glycosylated or phosphorylated. The protein was localized predominantly in the cytoplasm of HIV-2 infected cells. This HIV-2 p16 appears to be associated with the mature virion, but we do not know at present whether the protein is packaged inside the viral particles or if it is associated with the envelope of the virus during the budding process.

In summary, we identified a novel gene product of HIV-2 (p16) and generated reagents that may be used as diagnostic reagents as well as help elucidate the function of the p16 in HIV-2 infection and pathogenisis. The fact that the sera of HIV-1 infected people do not recognize the recombinant vpx protein in a Western blot assay could be

exploited to generate a sensitive assay to discriminate between people infected with HIV-1 or HIV-2.

In Vivo Expression of the SIV_{agm} Carboxy Terminus of Env

Since the carboxy terminus of the envelope transmembrane protein has been implicated in the cytopathic effect of HIV-1 in vitro, we decided to investigate whether putative expression of the open reading frame located after the termination codon correlates with the pathogenicity of SIV in vivo. Two synthetic peptides were generated from the inferred amino acid sequence of SIV and tested for reactivity by Western blot against the sera of naturally and experimentally infected monkeys as well as against sera of HIV-2 infected individuals. Results indicate that the protein synthesized from this open reading frame is expressed in vivo, since an immune response can be detected against the synthetic peptides in 2 out of 3 experimentally infected animals. However, no correlation can be found between its expression ar disease progression at this time. Furthermore, a rabbit immune serum raised against the synthetic peptide failed to identify any specific protein in SIV infected cells.

Based upon the hydropathy profile of the inferred amino acid sequence downstream from the termination codon (position 746-890), two hydrophilic regions were chosen for generating synthetic peptides (Fig. 11). The first peptide, THTQQDPALPTREGKEGDG, is located at the amino terminus and was designated PT-1 while the second, LRRIREVLRTELTY, was designated PT-2 (Fig. 11). The peptides were synthesized using solid phase peptide synthesis technology by Cambridge Biochemical Corporation. The synthetic peptides (1 ug each) were bound to nitrocellulose and reacted in a Western blot assay with sera obtained from various animals. An unrelated peptide of equivalent molecular weight was used as a negative control (WSKMDQLAKELTAE). Sera were reacted with nitrocellulose strips in a 1:100 dilution and the immune complexes were detected using iodinated Staphylococcus Aureus protein-A of 5×10^5 cpm/ml in blotto reagent (40). Sera obtained from animals (Table 8) which scored positive when tested by Western blot using SIV viral proteins were tested along with animals that scored negative in the Western blot assay.

Both synthetic peptides were recognized by serial sera obtained from a macaque (6325) 2 months after experimental infection with SIV (49). As shown in the lower part of Figure 12, no reactivity was detected in the sera obtained before the inoculation of the animal and 1 month after inoculation. Reactivity against PT-1 and PT-2 was detected two months after inoculation and lasted to the death of the animal from

immunodeficiency 17 months later. Animal 6324 recognized only one of the peptides (PT-1) (lower part of Fig. 12). The immune response against PT-1 also lasted till death from immunodeficiency 18 months later. In both macaques the reactivity against the synthetic peptides was coincident with the onset of antibodies against the major gag protein (p24-26) and the putative truncated transmembrane portion of the envelope protein (gp32-34) (upper part of Fig. 12). Animal 6325 also developed detectable antibody response against the extracellular portion of the envelope protein (gp120). In contrast, animal 6324 exhibited a lower titered immune response against the gp120. The third macaque (6323) sero-converted one month after SIV inoculation and developed a strong immune response to both the envelope glycoproteins and the gag proteins, but its serial serum samples did not recognize the peptides PT-1 and PT-2. However, the 6323 animal developed AIDS and died after 18 months. Three baboons, inoculated with SIV, developed an immune response against viral proteins including the gp32-34 as measured by Western blot analysis while their sera did not react with PT-1 and PT-2 peptides (data not shown). The infected baboons did not show laboratory or clinical signs of immunosuppression.

All 17 sera tested from African green monkeys were positive in Western blot against SIV proteins but did not react with the synthetic peptides (Table 9). Similarly, Western blot SIV seropositive and seronegative samples from antibodies of three talapoin monkeys and 5 HIV-2 positive human sera did not react with the synthetic peptides (Table 10). The lack of detection of antibodies in these species suggests that the amino acid sequences may be poorly conserved in the cross immunoreactive viruses infecting animals and humans tested. This hypothesis is supported by the finding of a lower degree of amino acid identity found in the human HIV-2 isolates HIV-2_{ROD}, HIV-2_{NIH-Z} and HIV-$2_{SBL/ISY}$ (18, 21, 50) in the region corresponding to the PT-1 and PT-2 peptides of SIV (Fig. 13). The termination codon in the SIV envelope is located immediately after the acceptor splice site of the transactivator gene of SIV (19). It is, therefore, possible that messenger RNA independent from the env transmembrane mRNA could be generated through a splicing mechanism that uses this splice site. To investigate whether a specific protein encoded by this region could be detected, a rabbit antiserum against the synthetic peptide PT-1 was generated. The reactivity of the anti-PT1 serum was tested against the metabolically labelled proteins of SIV and HIV-2_{NIH-Z} infected cell lines in radioimmunoprecipitation (RIP) assay and in Western blot of unlabeled proteins from the same cell lines. While the sera from SIV infected monkeys did recognize specific viral proteins in the RIP assay of ^{35}S metabolically labeled protein of the SIV infected KW1 cell line, the anti-PT1 serum failed to immunoprecipitate specific protein(s)

(Fig. 14, first panel). Similar data were obtained when the RIP assay was performed on HIV-1 and HIV-2 infected cells. The anti-PT1 serum also failed to detect specific protein in a Western blot on cellular lysate of SIV and HIV-2 infected cells (data not shown).

DNA Sequence Determination of HIV-$2_{SBL/ISY}$

A recombinant lambda phage containing the complete provirus of HIV-2 was isolated from a genomic library constructed from the DNA of SBL6669 infected human T-cell line Hut78, using the SIV gag (B16) and envelope probes (SS35) (45). A recombinant clone designated HIV-$2_{SBL/ISY}$ was purified and the inserted DNA was used to generate several subclones in the Bluescript Vector (44). The restriction enzyme pattern of the HIV-$2_{SBL/ISY}$ clone, as presented in the upper part of Figure 15 differs considerably from that of previously analyzed HIV-2 proviruses designated HIV-2_{NIH-Z} (21), and HIV-2_{ROD} (18) and SIV_{MAC} (20, 51). The complete DNA sequence of HIV-$2_{SBL/ISY}$ has been determined by the dideoxy chain termination method of Sanger using Sequenase (46, 52), and the chemical modification/cleavage method of Maxam and Gilbert (47). A 10% divergence has been estimated to exist between HIV-$2_{SBL/ISY}$ and HIV-2_{NIH-Z} or HIV-2_{ROD}. Thus, the HIV-2 isolates display a considerable degree of variation, similar to that found among the isolates of HIV-1.

Biological Activity of Recombinant HIV-$2_{SBL/ISY}$

The recombinant phage clone, HIV-$2_{SBL/ISY}$ was transfected into the human neoplastic cell line Hut78. The supernatant of the cell culture was found positive for magnesium dependent reverse transcriptase one week after transfection. Viral expression was confirmed by immunofluorescent straining of the infected cells using HIV-2 positive serum. Genomic DNA was isolated from the infected cells, restricted with endonucleases, electophoresed and blotted according to standard procedures (44, 48). Southern blot analysis of the total genomic DNA isolated from the infected cell line indicated the presence of viral sequences (bottom of Figure 15). Hybridization of Xba I and Eco RI cleaved DNA's to the SIV gag gene probe (B16) revealed the same internal bands for the uncloned parental HIV-$2_{SBL6669}$ and the HIV-$2_{SBL/ISY}$ proviral DNA, indicating that HIV-$2_{SBL/ISY}$ is representative of the majority of the genotype present in the parentally infected cell line. Different restriction enzyme patterns were observed with the genomic DNA isolated from the SIV and the HIV-2_{NIH-Z} infected cell lines. Electron microscopic analysis performed on the HIV-$2_{SBL/ISY}$ transfected cells

revealed the presence of mature virions with the expected cylindrical shaped core typical of lentiviruses (Figure 16) and budding particles from the cell membrane (see inset of Figure 16), indicating that the transfection of the HIV-$2_{SBL/ISY}$ DNA induced a productive infection of the Hut78 cell line.

Immunological Characterization of the HIV-$2_{SBY/ISY}$

Western blot analysis and radioimmunoprecipitation were performed on the viral particles obtained from infected Hut78 cells (Figure 18). The nitrocellulose strips containing unlabeled virion proteins were reacted with a sera from an SIV_{mac} experimentally infected monkey (Figure 17, lane 1), an HIV-2 infected individual (Figure 17, lane 2) and a normal donor (NS) as well as a mouse monoclonal antibody directed against the HIV-2/SIV_{mac} major gag protein (p24-26), and control ascites fluid (C). The most reactive and apparently most abundant viral proteins detected in the HIV-$2_{SBL/ISY}$ and SIV_{mac} virions were the gag p24-26 and p15 proteins (see the first two panels of Figure 17). Similar results were obtained when radiolabeled HIV-$2_{SBL/ISY}$ virion proteins were used in radioimmunoprecipitation (see left panel of Figure 17). The envelope glycoprotein gp120 was barely detected by immunoprecipitation and not at all by Western blots (Figure 17). The DNA sequence of the replication competent proviral clone lacks a termination codon in the transmembrane portion of the envelope gene and should yield a transmembrane envelope glycoprotein of around 40 Kd. A very faint band located around 40 Kd could be detected in RIP or Western blot assays of HIV-$2_{SBL/ISY}$ using positive human sera. However, a well characterized specific antiserum will be needed to clearly define this protein band. A smear, probably representing proteins with different relative migration rates were detected around 30 Kd in SIV_{mac}. This smear has been interpreted as the truncated form of the transmembrane protein (53) although the amino acid sequence after the termination codon is expressed in infected animals (54). A smear could also be detected in the same region in HIV-$2_{SBL/ISY}$ using the human serum from a patient infected with HIV-2.

Host Range and Cytopathic Effect of HIV-2

The Hut78 cell line producing the HIV-$2_{SBL/ISY}$ was expanded and virus was concentrated from 10 liters of supernatant as described (55). The HIV-2_{NIH-Z} isolate was used in a parallel experiment to infect the same cell lines. Equal amounts of

concentrated virus were then used to infect several human cell lines. Replication and propagation of the virus was monitored by reverse transcriptase assay of the culture supernatant and immunofluorescence on fixed cells. The biological effect exerted by the HIV-2 isolates on the infected cells was measured by counting the number of viable cells and syncytia at different time intervals. The results on the infectivity of the HIV-2 isolates are reported only for the HIV-2$_{SBL/ISY}$ isolate (Table 9). HIV-2$_{SBL/ISY}$, as well as HIV-2NIH-Z infected the HTLV-I transformed T-cell line MT-2, the T-cell clone 55 immortalized by a single defective copy of HTLV-I (56) and the CEM, Hut78, Molt-3, H9 and U939 neoplastic cell lines. The highest cytopathic effect, exerted by both HIV-2$_{SBL/ISY}$ and HIV-2$_{NIH-Z}$ was observed in the HTLV-I infected cells and in the H9 cells (Table 9), and is coincident with the highest number of syncytia present in the cell culture (Figure 18). The parental virus HIV-2$_{SBL6669}$ also infected Hut78, U937 clone 16, CEM, and Jurkat T-cells (Table 10), with the highest cytopathic effect observed on the Jurkat and U937-16 cell lines (Table 10).

d) Discussion

Most of the viral gene products that regulate viral expression and replication of HIV-1 are also present in HIV-2. In fact, the putative functional domains of the regulatory proteins are evolutionary conserved. However, differences in the overall structure of the HIV-2 LTR's which are larger than HIV-1 LTR's account for a variation in the responsive region to the viral transactivator gene (tat) (57). More genetic information is also needed to encode the tat and rev proteins as reflected by the study of functionally active SIV and HIV-2 cDNA's (11). However, the major structural differences appear to be the presence of a gene (vpu) in type 1 viruses (19) and another gene (vpx) which is present only in type 2 viruses (58, 59). The amino acid sequences of these two genes are not homologous and whether they are functionally equivalent is still an open question.

SIV$_{mac}$ and HIV-2 viruses also differ from HIV-1 in the length of the transmembrane portion of the viral envelope. Whereas HIV-1 encodes for a gp41, SIV$_{mac}$ contain a translation termination codon reducing the transmembrane protein to 32 Kd (gp32). In addition, some isolates of HIV-2 also appear to have a truncated transmembrane protein (18, 20, 21, 51). This work shows that the region after this termination codon is expressed and immunogenic in SIV infected monkeys. Two out of three experimentally infected macaques showed reactivity against one or both synthetic peptides, PT-1 and PT-2. No definitive correlation between reactivity against PT-1 and

PT-2 and disease progression could be found since all three macaques died of AIDS from 17 to 18 months following infection. It is intriguing that 3 SIV inoculated baboons did not mount an immune response to the generated peptides. These baboons were SIV virus positive and antibody positive although they remained healthy 2-1/2 years after inoculation. This may suggest an in vivo modulation of expression of the termination codon that is correlated with pathogenic effect.

No reactivity against PT-1 was detected in the other SIV-infected animals studied or in HIV-2 infected humans. As genetic variability occurs among the various isolates of HIV-1 and HIV-2, it is likely that different monkey species are infected with related SIV viruses with slightly different amino acid sequences in this region. This assumption is underscored by the fact that the region in the transmembrane protein after the termination codon is one of the least conserved regions between SIV and different HIV-2 isolates (57, 58, 59). Likewise, the lack of immune reactivity in HIV-2 infected humans may well be the result of a different amino acid sequence in the region of HIV-2 homologous to the region in SIV from which PT-1 and PT-2 were derived.

It remains to be resolved why, despite apparent expression of the region after the termination codon in vivo, the SIV_{mac} infected cells appear to express only the truncated transmembrane protein, gp32. Since the virus inoculum to experimentally infect the macaques contains the progeny of more than 1 viral clone, we could assume that there are viral genotypes in this population not containing the termination codon and that are expressed and immunogenic in vivo. Expression of the complete gp41 may, in this case, generate immune reactivity against PT-1 and PT-2. An alternative explanation could be that the termination codon is suppressed in vivo. Finally, the presence of the tat splice acceptor site, right after the stop codon, would make it possible to express the region containing PT-1 and PT-2 independently from the transmembrane protein. Since no proteins could be detected with the rabbit antiserum raised against PT-1, this issue can not yet be resolved.

The availability of a replication competent HIV-2 proviral clone provides the tools to study this newly identified gene (vpx) as well as the role of the "non-essential" accessory genes of HIV in virus replication and the relevance of the structural differences in vivo. Furthermore, the regulatory elements of the viral LTRs and their interaction with the regulatory proteins can be evaluated in the context of a complete infectious genome. The value of an infectious HIV-2 clone in developing an animal model for HIV vaccine and therapy studies should also be emphasized. Rapid progress in the development of a protective vaccine against HIV has been impaired by the lack of a suitable and cost effective animal model. Successful infection of non-human primates has

been achieved only in chimpanzees (60) and gibbons (61) which are scarce. Furthermore, no pathogenicity of the virus is observed in these animals. Since the parental HIV-2$_{SBL6669}$ and the molecular clone HIV-2$_{SBL/ISY}$ productively infect rhesus macaques (our unpublished results) and these macaques are sensitive to the pathogenic effect of HIV-2, the development of an animal model using a highly related human virus will obviously be very valuable. Finally, we have recently shown that individual isolates of HIV-1 are composed of microvariants with distinct biological properties and susceptibility to given neutralizing sera (62). The composition of this population presumably drifts due to new mutations and selection in response to changes in available target cells and host immunity. The availability of an infectious molecular clone will allow us to measure the genetic evolution of the viral genome and its immunological consequences in the infected host.

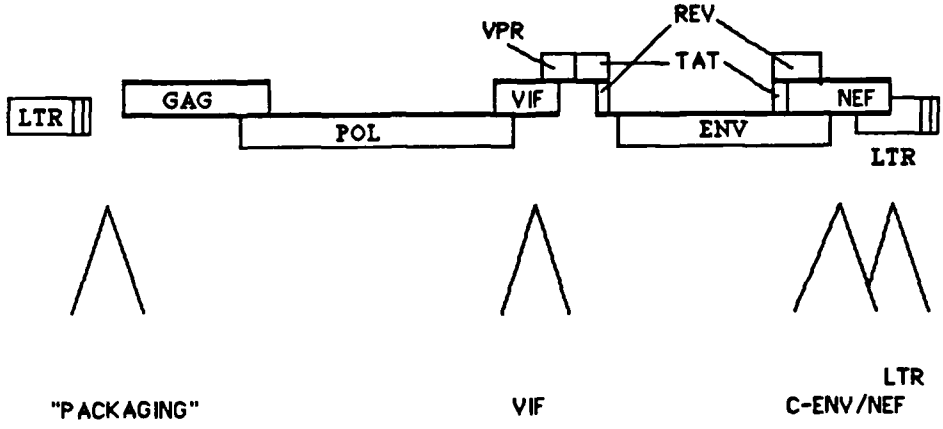


Fig. 1 Deletion Mutants of HIV-1. Deletion mutants in various portions of the HIV-1 genome were generated by Bal 31 digestion followed by religation, or by site-directed mutagenesis. Shown above are the locations of the mutations in the 5' region, the vif, the C-env/nef and the LTR regions for studying effects on packaging, transmission cytopathicity and transactivation, respectively.

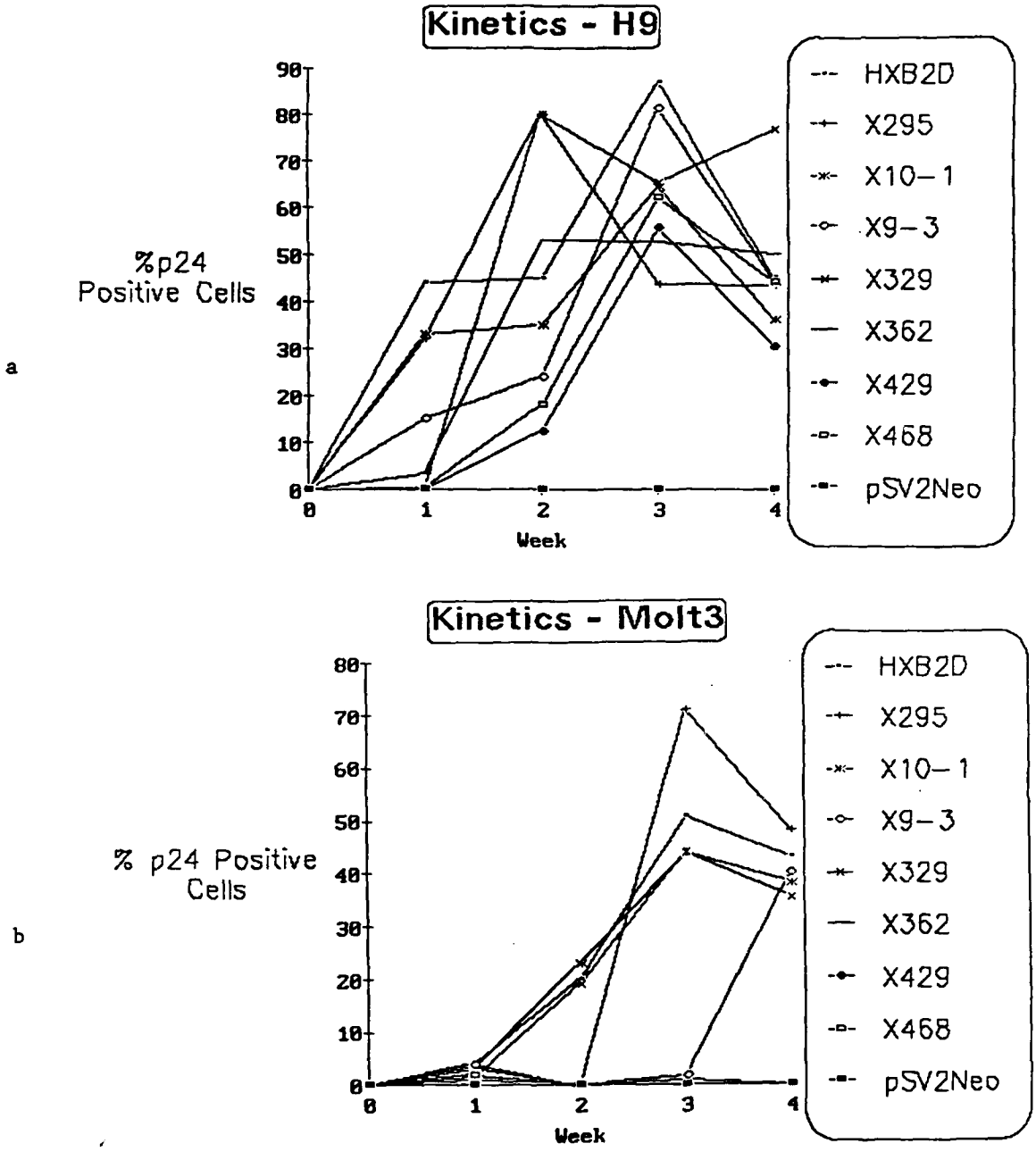


Figure 2. Transmission kinetics of deletion mutants in the carboxy region of env. Cos-1 cells were transfected with 5 µg of DNA from pHXB2D, pSV2neo, or deletion mutants. Transfected cells were incubated with 5 x 10^5 polybrene trated H9 or Molt-3 cells and maintaintd in RPMI-20%FCS. Weekly examination of viral transmission was carried out by indirect immunofluorescence with anti gag p24 monoclonal antibody.

a. Kinetics of viral transmission to H9 cells.

b. Kinetics of viral transmissson to Molt-3 cells.

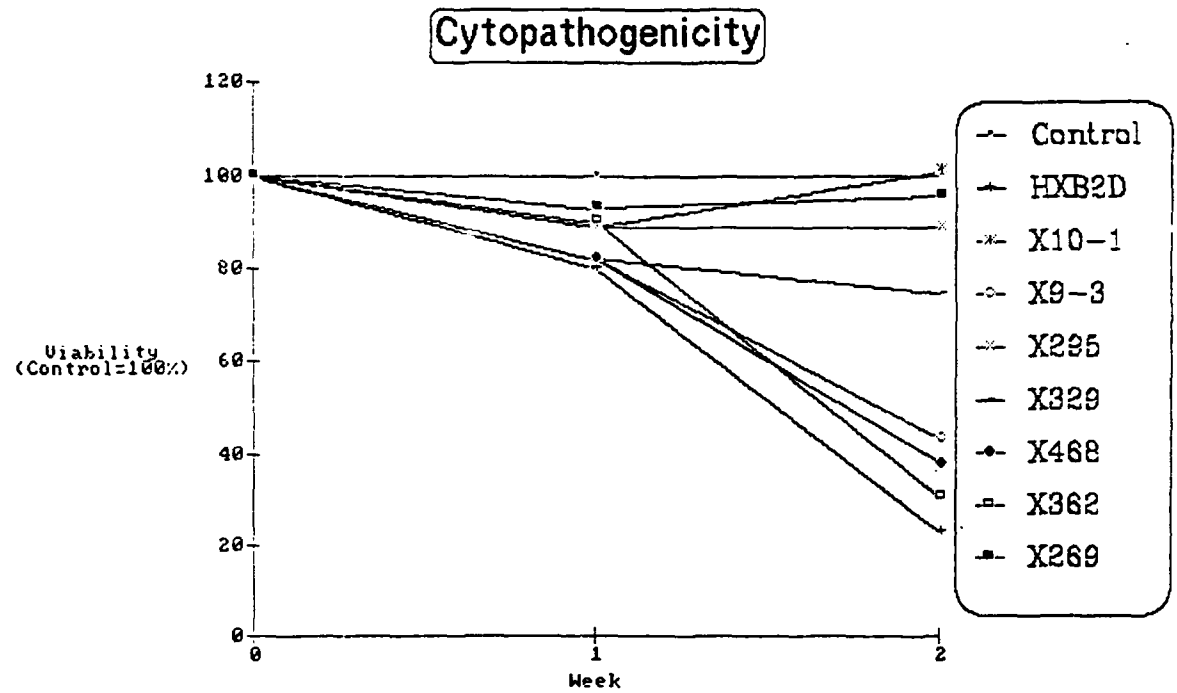


Figure 3. Cytopathogenicity of cell free virus preparations of deletion mutants. Polybrene treated H9 cells (3 x 10^6) in 3 ml RPMI-20% FCS were incubated with 300 x TCID50 of the respective virus preparations (or media as the control) for 1 hour at 37° C. The cells (100µl) were transfered to triplicate wells in 24-well microtiter plates and 2 ml of fresh medium was added to each well. Plates were fed with 1 ml fresh medium weekly. Cell viability was assessed weekly by exclusion dye 2% trypan blue.

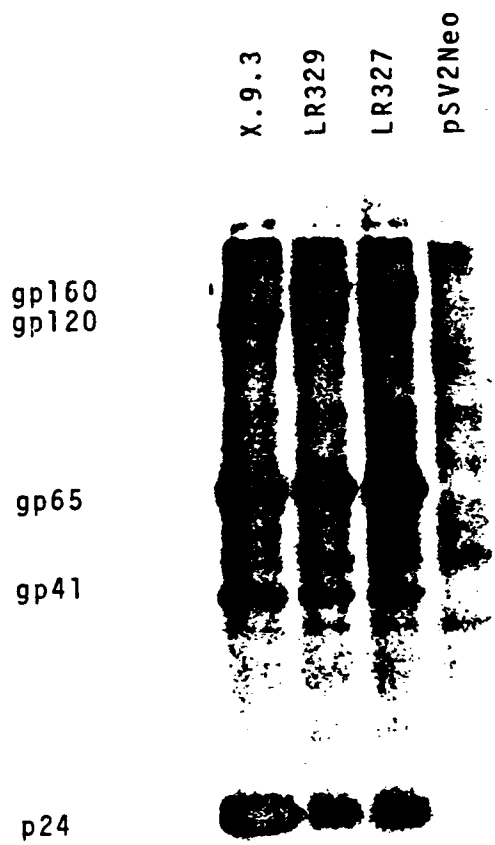


Figure 4. Radioimmunoprecipitation of ^{35}S-methionine labeled extracts of H9 cells infected with deletion mutants in the carboxy region of env. H9 cells were infected with mutant viruses from transfected Cos-1 cells and labeled with ^{35}S-methionine overnight. Cell extracts were prepared, reacted with human anti-HIV-1 sera, collected by Sepharose A chromatography, and analyzed on polyacrylamide gels.

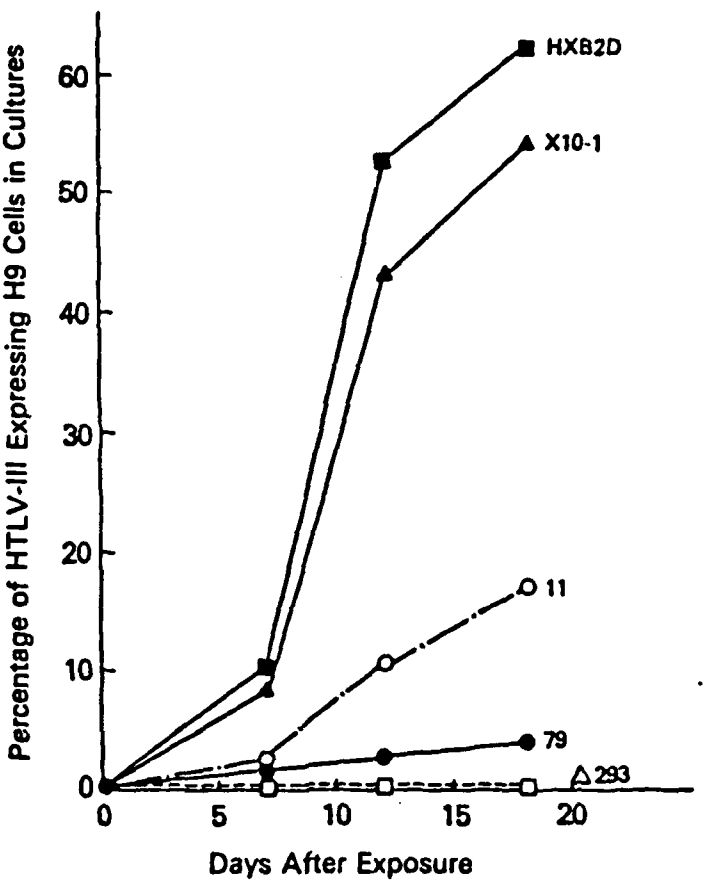


Figure 5. Transmissibility of "packaging" mutant viruses into H9 cells. Plasmid DNA containing the deletions in the sequences between the 5'LTR and gag (Δ293, #79, and #11) were transfected into Cos-1 cells. The resulting supernatants were tested for the presence of infectious virus by cocultivating with polybrene treated H9 cells and monitoring the percentage of cells positive for HIV-1 p24 by immunofluorescence. Plasmids HXB2D and X10-1 were included as positive controls.

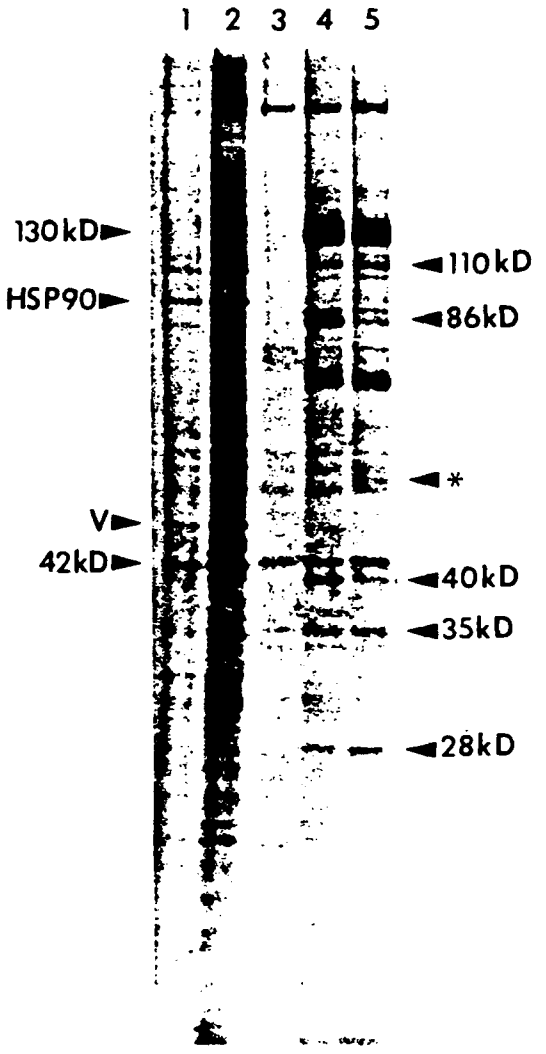


Figure 6. Microscale affinity assay of proteins binding to the HIV enhancer sequence. Nuclear extracts of cells labeled with ^{35}S-methionine were prepared and incubated with biotinylated oligonucleotides containing the enhancer sequence. The protein/oligonucleotide complexes were captured on streptavidin beads. The specific binding proteins were eluted and analyzed on polyacrylamide gels. Lanes 1 and 2 are two different amoutns (1/1000 and 1/200, respectively) of the crude nuclear extract used for each reaction. Lane 3 represents the proteins that are recovered when all the ingredients of the reaction except biotinylated probe are added. Lane 4 represents the proteins recovered with the biotinylated HIVEN3c1/2 oligonucleotide, and Lane 5 the proteins recovered with the biotinylated HIVEN3m3/4 oligonucleotide. Exposure was for 2 days on Kodak XAR film.

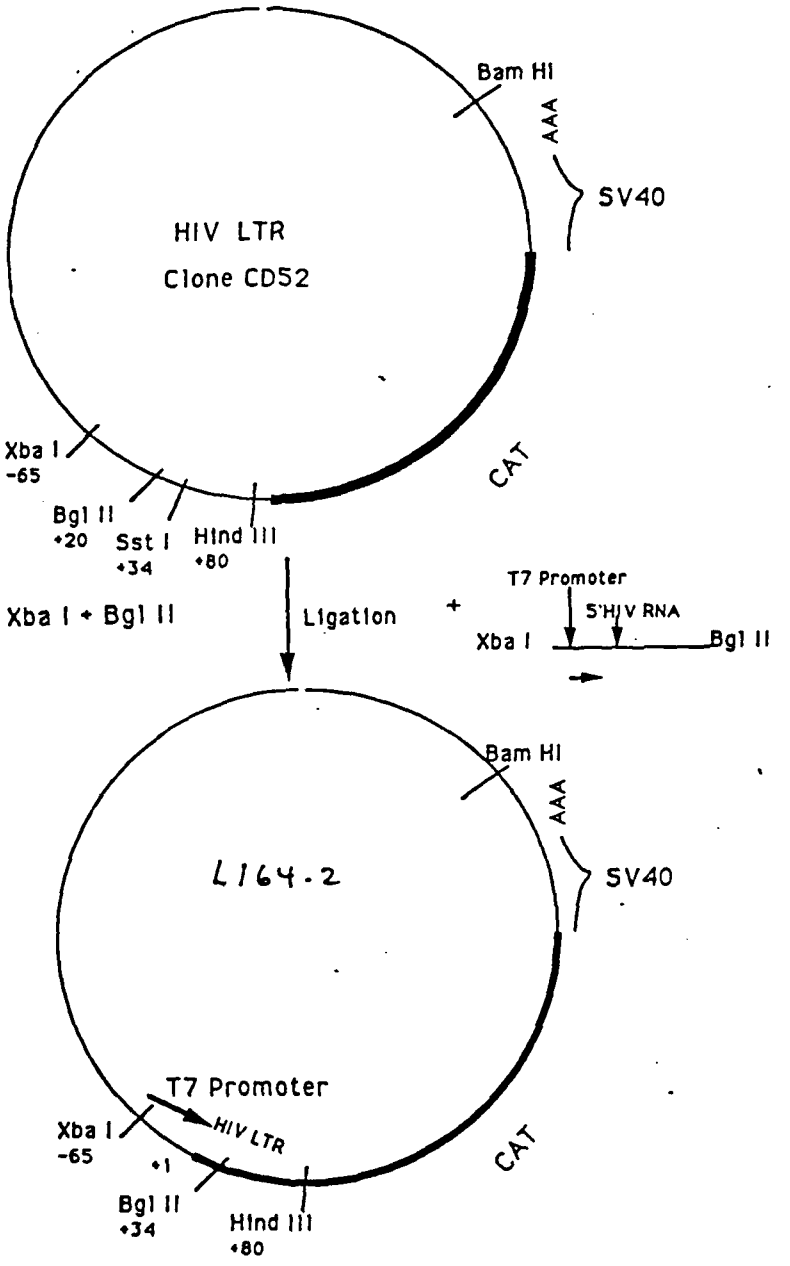


Figure 7. Construction of Plasmid L164.2. . Plasmid L164.2 was constructed from clone CD52 which containted the HIV-1 LTR gene linked to the Chloramphenicl amino transferase gene (CAT) and the the polyadenylation site of SV40. The T7 promotor was inserted upstream of the TAR region by digestion with Xba I and Bgl II and ligation of a Xba I/Bgl II fragment containing the T7 promoter linked to the 5' region of HIV.

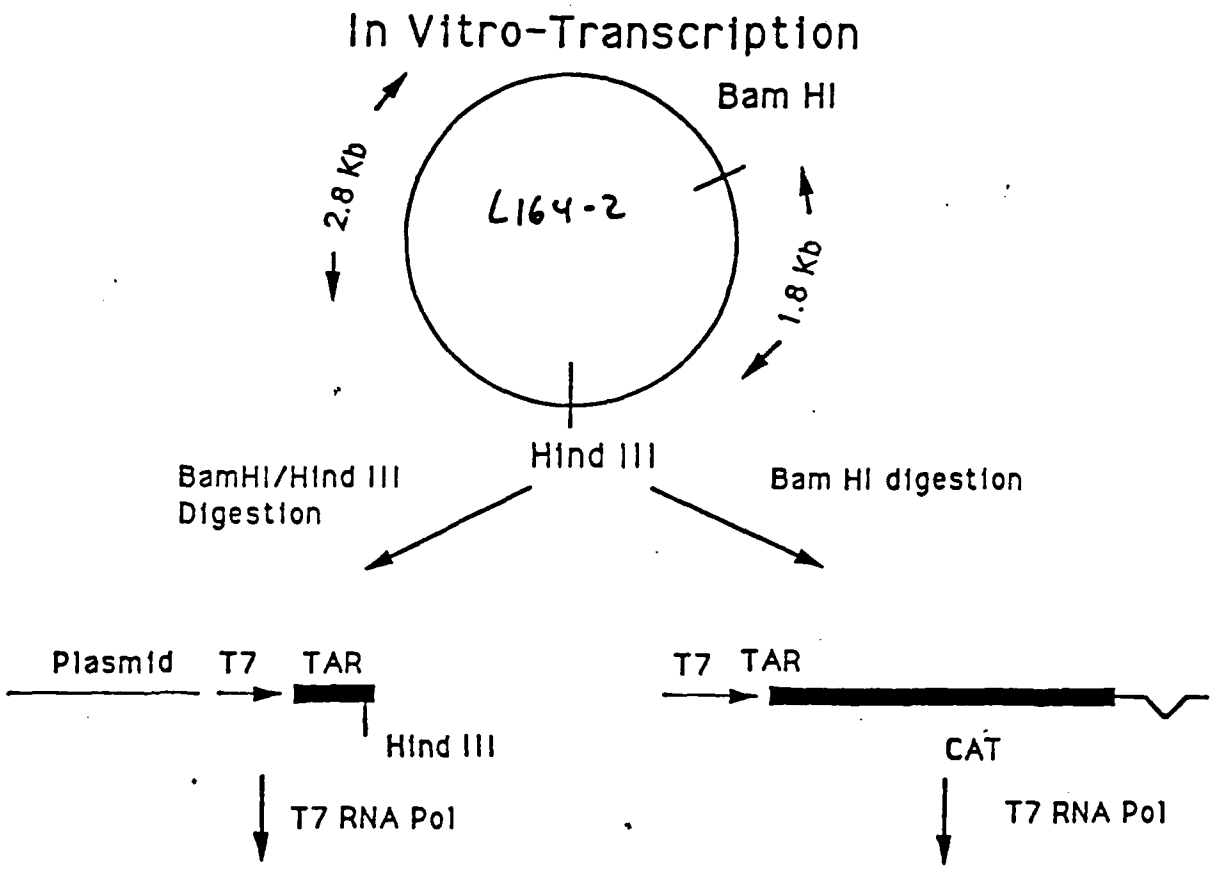


Figure 8. In vitro transcription of L164.2. The L164.2 plasmid was linearized by digestion with Bam HI. The resulting DNA was used as a template for T7 RNA polymerase to transcribe mRNA containing the CAT gene linked to the TAR sequences.

GENOMIC STRUCTURE OF HUMAN AND NON HUMAN PRIMATE RETROVIRUSES

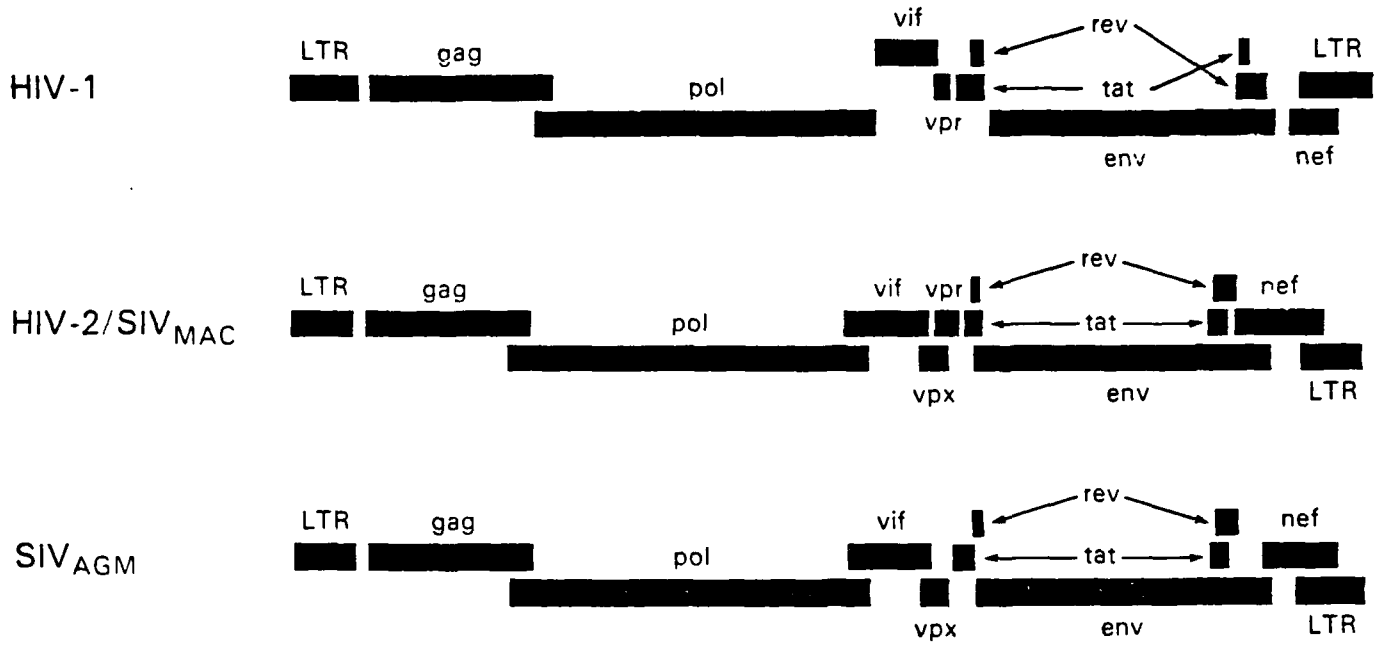
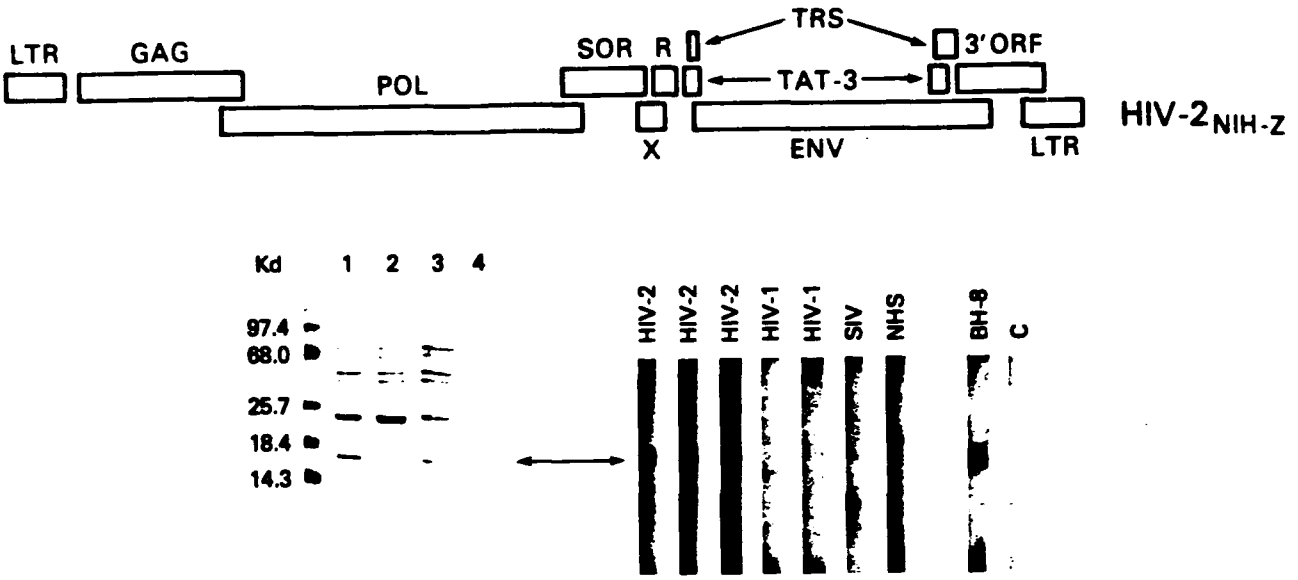


FIGURE 9: Genomic Organization of HIV-1, HIV-2/SIV$_{mac}$, and SIV$_{agm}$.

The bars represent open reading frames corresponding to the various genes, with the exception of the LTRs.

gag = core proteins, vif = virus infectivity gene,
pol = polymerase gene, vpr = unknown function,
vpx = p16, unknown function, tat = p14, transactivation gene.
rev = p19, regulatory gene, env = envelope protein,
nef = p27, retrovirus inhibitory factor
LTR = long terminal repeat unit.



Source	N°Tested	N°Positive	%Positive
HIV-2 Infected Humans	26	11	42
HIV-1 Infected Humans	8	0	0
SIV Infected Macaques	5	0	0
Uninfected Humans	4		
Total	44		

Figure 10: Analysis of the HIV-2 vpx Gene.

The right panel of the figure represents a SDS/PAGE gel of total E. coli proteins stained with Coomassie blue. Lanes 1 to 3 contain protein lysates from E. coli transfected with the vpx (X-orf) of HIV-2NIHZ in the REV expression vector. As a negative control E. coli were transfected with the REV vector (lane 4). A specfic band with a relative migration corresponding to a molecular weight of approximately 17 Kd which is indicated by an arrow. The left part of the figure represents an autoradiogram of a Western blot performed on the total E. coli protein lysate containing the recombinant vpx (X-orf) with human sera from HIV-1 and HIV-2 infected individuals, normal human serum (NHS), monkey serum from an animal infected with SIV, and mouse monoclonal antibodies (BH8) directed against the vector epitopes which are located at the amino terminus of the recombinant protein and a mouse antibody control (C). The arrow indicates that the 17 Kd band reacts with the HIV-2 postive human sera.

The recombinant protein for vpx was analyzed by immunoblot using sera from HIV-1 and HIV-2 seropostive humans, SIV seropositive monkeys, and normal human control sera. The 17 Kd band was recognized by 42% of the HIV-2 infected human (table above) and none of the others.

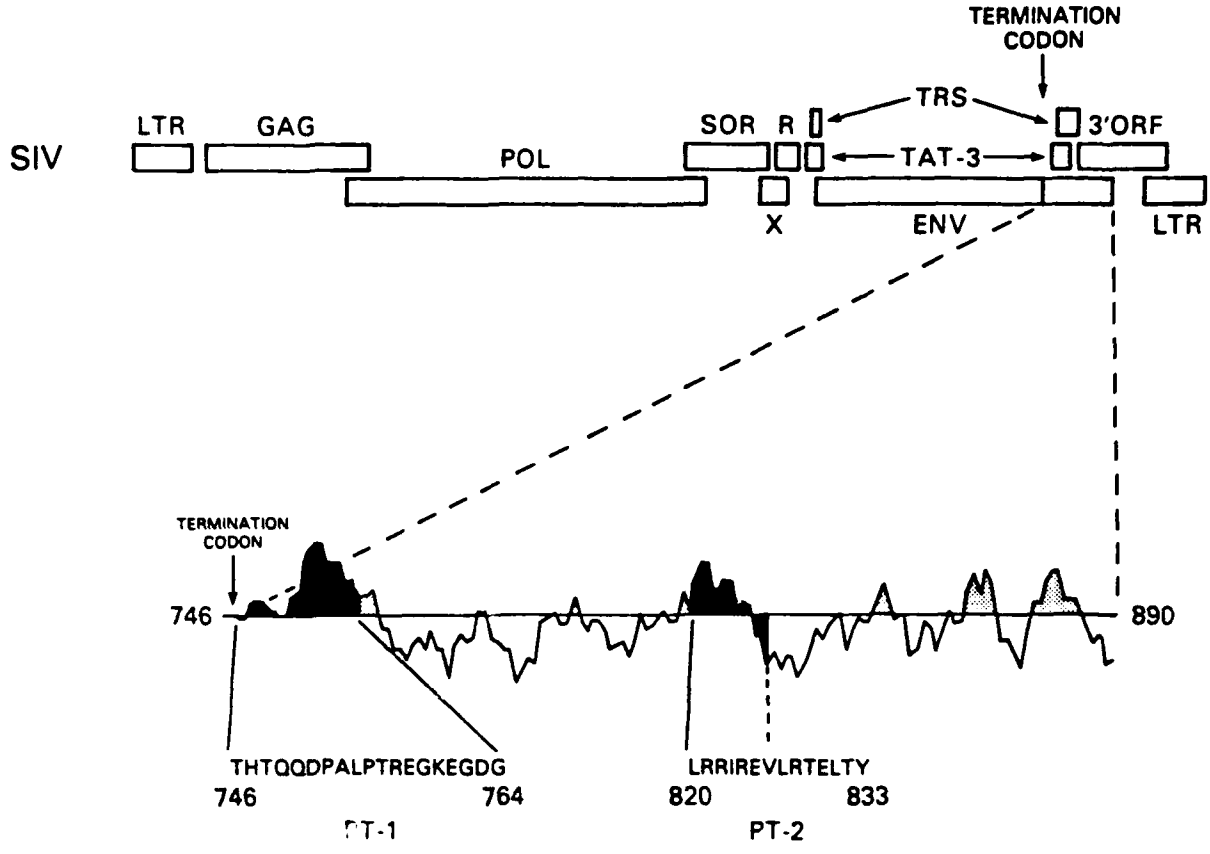


Figure 11: The SIV Genome and the Regions used to make Synthetic Peptides.

The upper part of the figure represents the genome of SIV along with the relative positions of the various genes. The position of the termination codon in the transmembrane portion of the envelope is indicated by the arrow. The lower part of the figure shows the hydropathy profile of the region after the termination codon. The dotted areas are hydrophilic; whereas, the clear areas are hydrophobic. The darken areas are regions used for PT-1 and PT-2 peptides.

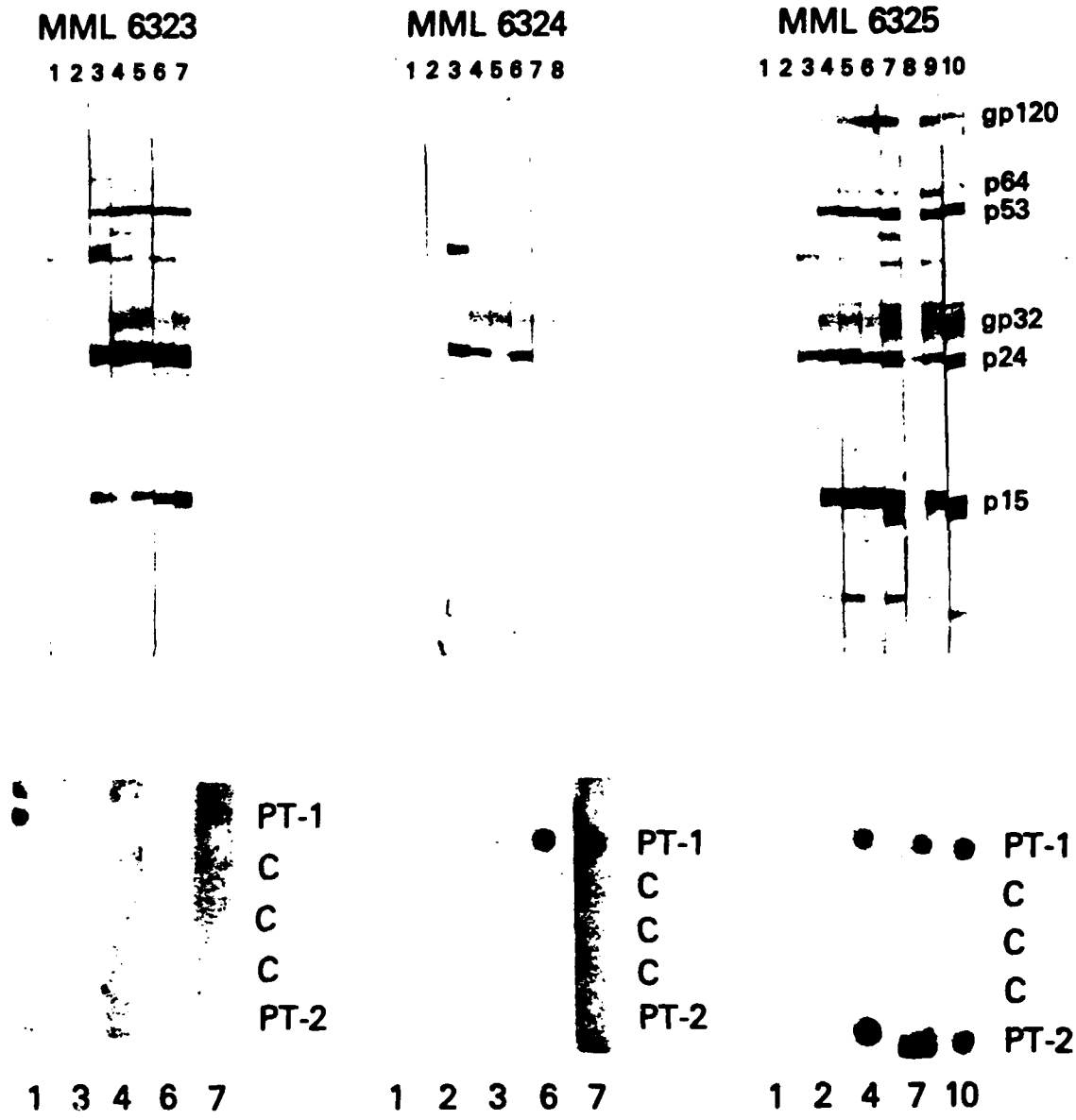


Figure 12: Western Blot Analysis of Sera Obtained from Rhesus Macaques.

Three rhesus macaques were inoculated with SIV (MML 6323, MML 6324, and MML 6325). In the upper part of the figure the sera were reacted with nitrocellulose strips containing total SIV proteins. Viral proteins were obtained from the same SIV strain that was used to inoculate the animals. The lower portion of the figure shows results obtained when sera from the same three animals were reacted with the synthetic peptides PT-1 and PT-2 (1 ug) and the control peptide (1, 2, and 3 ug). The numbers on the abscissa indicate the date that the sera were obtained following infection as expressed in months.

PT-1

SIV	T	H	T	Q	Q	D	P	A	L	P	T	K	E	G	K	K	G	D	G
	.		.	.	.		.	.	.		.	.		.	.	.	.		.
HIV-2$_{NIH-Z}$	I	H	I	H	K	D	Q	E	Q	P	A	R	E	E	T	E	E	D	V
	.		.	.	.		.	.	.		.	.		.	.	.	.		
HIV-2$_{ROD}$	I	H	I	H	K	D	R	G	Q	P	A	N	E	E	T	E	E	D	G
	.		.	.	.		.	.	.		.	.		.	.	.	.		.
HIV-2$_{SBL/ISY}$	I	H	I	H	K	D	W	E	Q	P	D	R	E	E	T	E	E	D	V

PT-2

SIV	L	R	R	I	R	E	V	L	R	L	E	L	T	Y
		.	.			.	.				.	.	.	
HIV-2$_{NIH-Z}$	L	T	A	I	R	D	W	L	R	L	K	A	A	Y
	.	.	.	.		.	.					.	.	.
HIV-2$_{ROD}$	Y	Q	N	L	R	D	W	L	R	L	R	T	A	F
		.	.	.		.	.				.	.	.	
HIV-2$_{SBL/ISY}$	L	Q	P	L	R	D	W	L	R	L	K	A	A	Y

Figure 13: Comparison of Amino Acids Among Human HIV-2 Isolates.

Amino acid alignment of the SIV sequences which correspond to PT-1 and PT-2 with the equivalent sequence of three HIV-2 isolates. The dots indicate the diversity in amino acid composition.

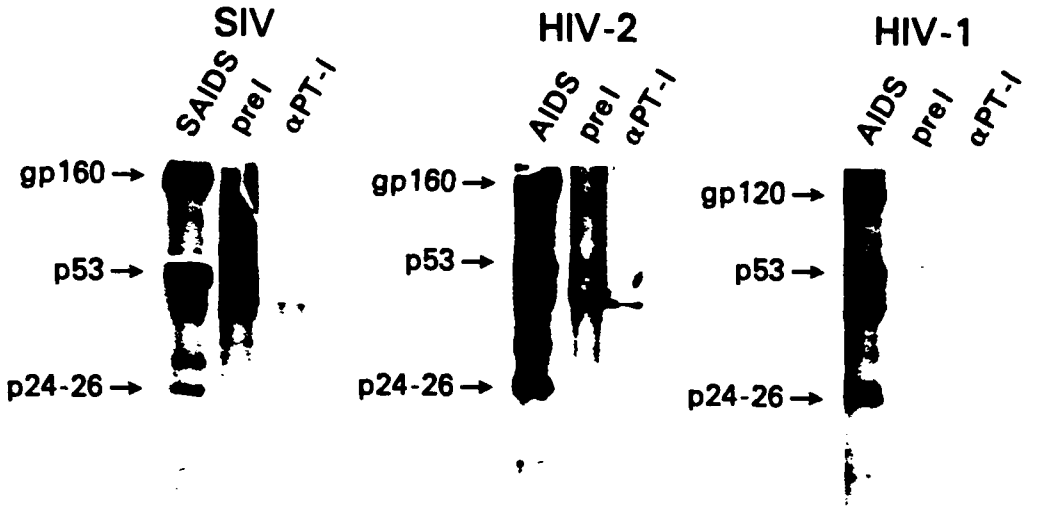


Figure 14: Reactivity of Rabbit Antiserum Against the Synthetic Peptide PT-1.

Immunoprecipitation of metabolically labeled total ceelular proteins from the SIV- infected human T cell line, HUT 78. SAIDS: serum from macaque 6325 (bleed 10) which was infected with SIV; AIDS: serum from patients with acquired immunodeficiency syndrome; preI: rabbit preimmune serum; anti-PT-1: immune serum raised against the synthetic peptide PT-1.

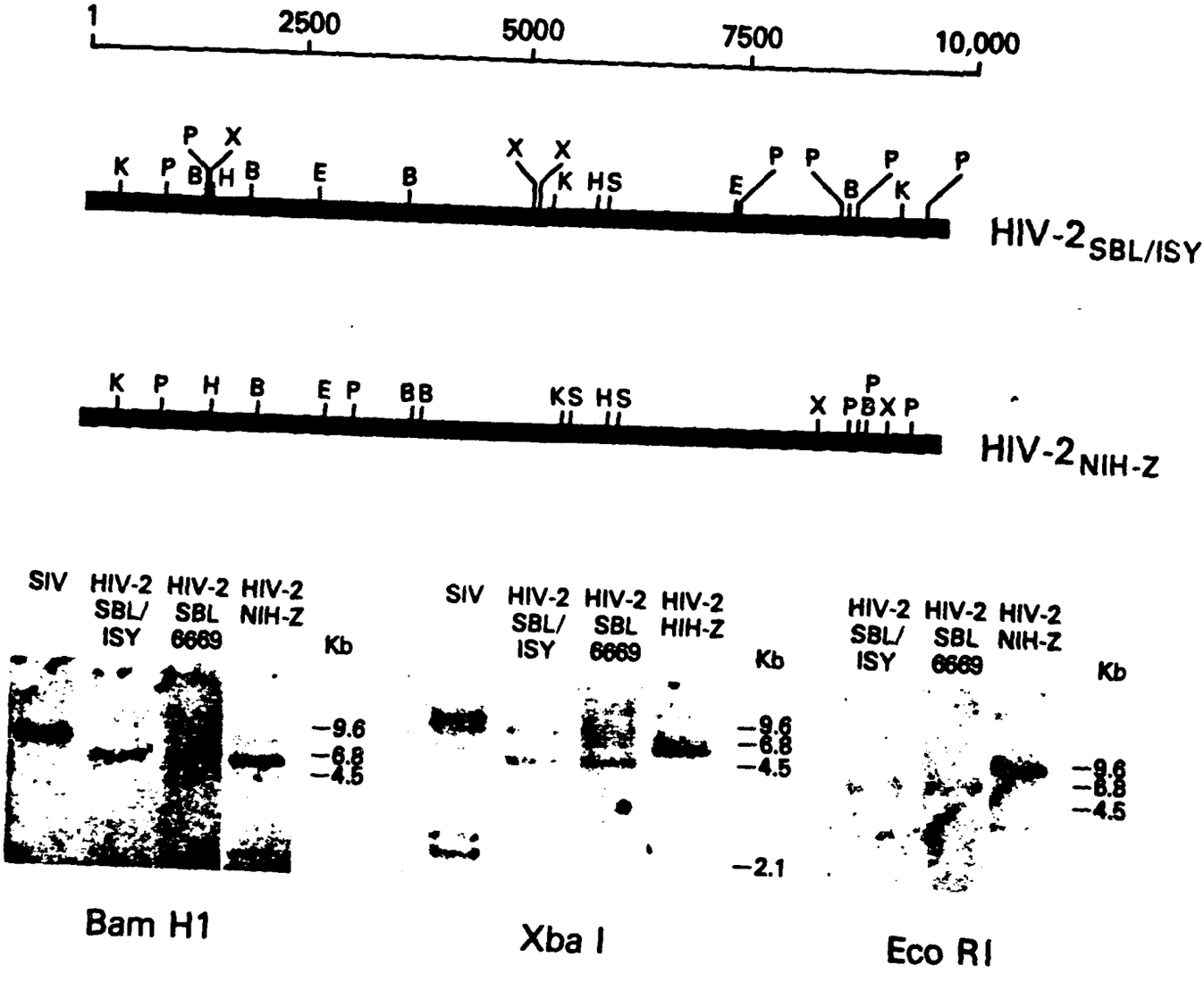


Figure 15: Restriction Enzyme Analysis of the Genomic DNA of HIV-2 Infected Cell Line, HUT 78.

The upper portion of the figure represents the endonuclease restriction maps of the proviral HIV-2 clones obtained from the viral isolates HIV-2_{NIH-Z} and HIV-$2_{SBL6669}$. The lower protion represents the results of Bam HI, Xba I, and Eco RI cleavage of the genomic DNAs of the HIV-2_{NIH-Z}, HIV-$2_{SBL6669}$, and SIV_{MAC251} infected cell lines. The second lane of each panel represents the analysis of DNA from the HUT 78 cell line transfected with the proviral clone HIV-$2_{SBL/ISY}$. K = Kpn I; P = Pst I; B = Bam HI; X = Xba I; H = Hind III; E = Eco RI; S = Sac I.

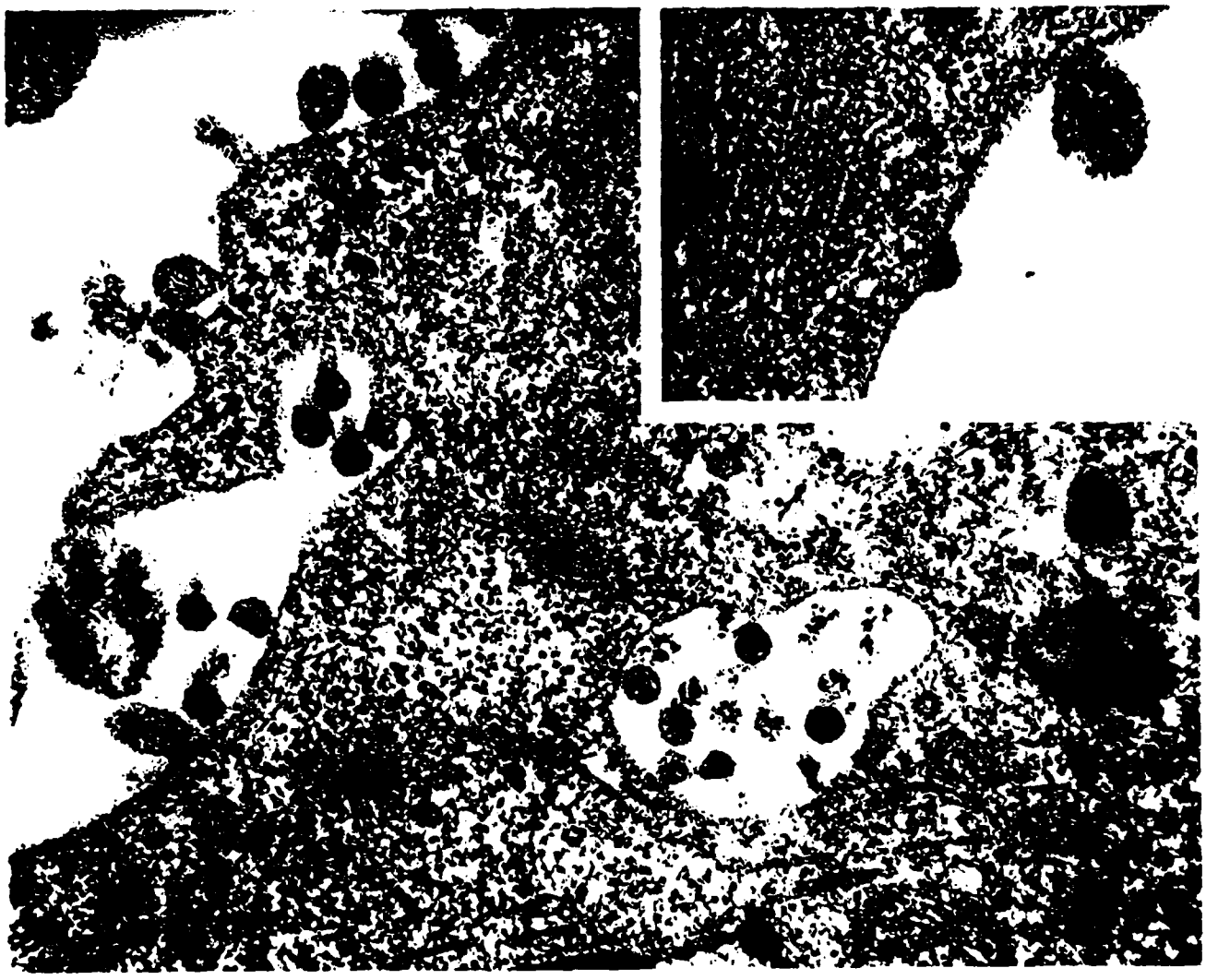


Figure 16: Electron Micrograph of the HIV-2$_{SBL/ISY}$.

The inset includes a section in which a budding viral particle from a HUT 78 cell can be detected. Several mature virions with dense cylindrical or round core can be seen in the remainder of the figure.

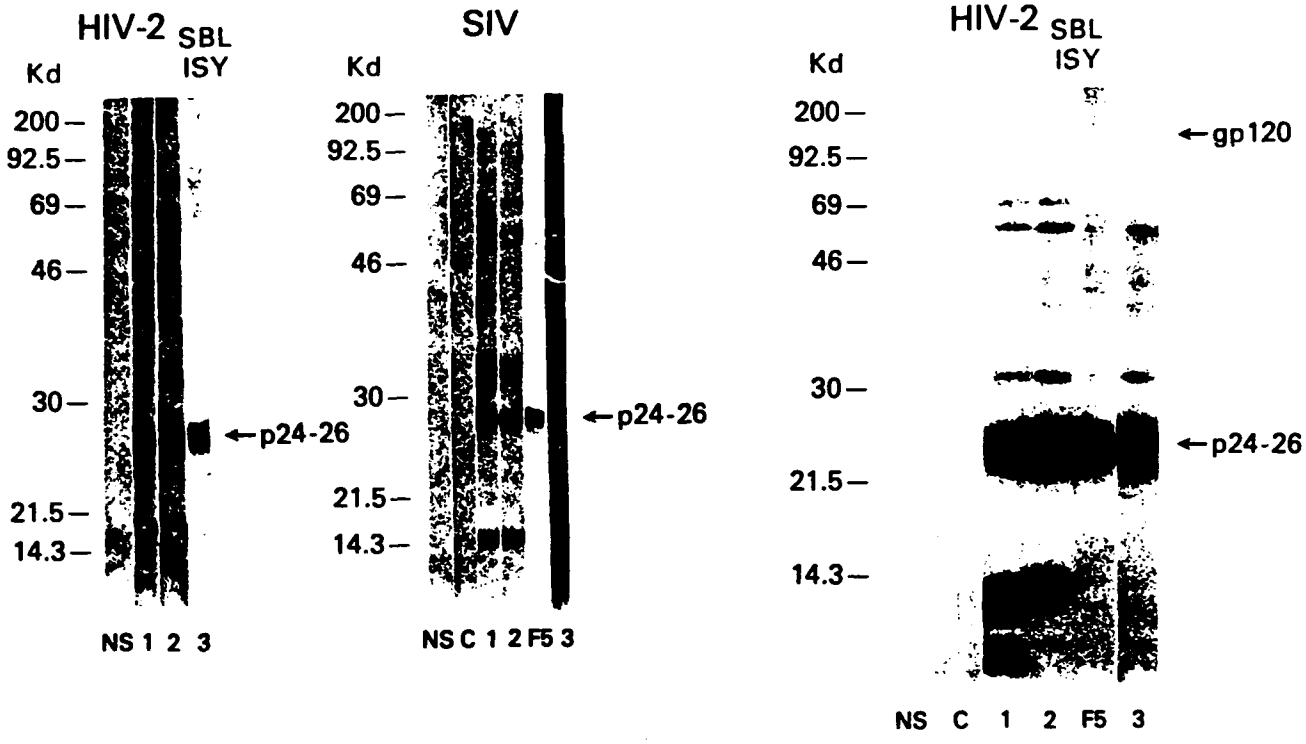


Figure 17: Western Blot Analysis of Viral Proteins.

The left and central panels represent the Western blot analyses of total viral proteins obtained from disrupted HIV-2$_{SBL/ISY}$ and SIV virions, respectively. The right panel represents and immunoprecipitation of metabolically labeled HIV-2$_{SBL/ISY}$ virions. NS = normal human serum; 1 = serum from a macaque experimentally infected with SIV; 2 and 3 = sera from human infected with HIV-2; c = control mouse ascite; F5 = mouse monoclonal antibody directed against the p24 of SIV. The molecular weight of the proteins was calculated with respect to the migration of standard markers from BRL.

CYTOPATHIC EFFECT OF HUMAN IMMUNODEFICIENCY VIRUSES TYPE 2

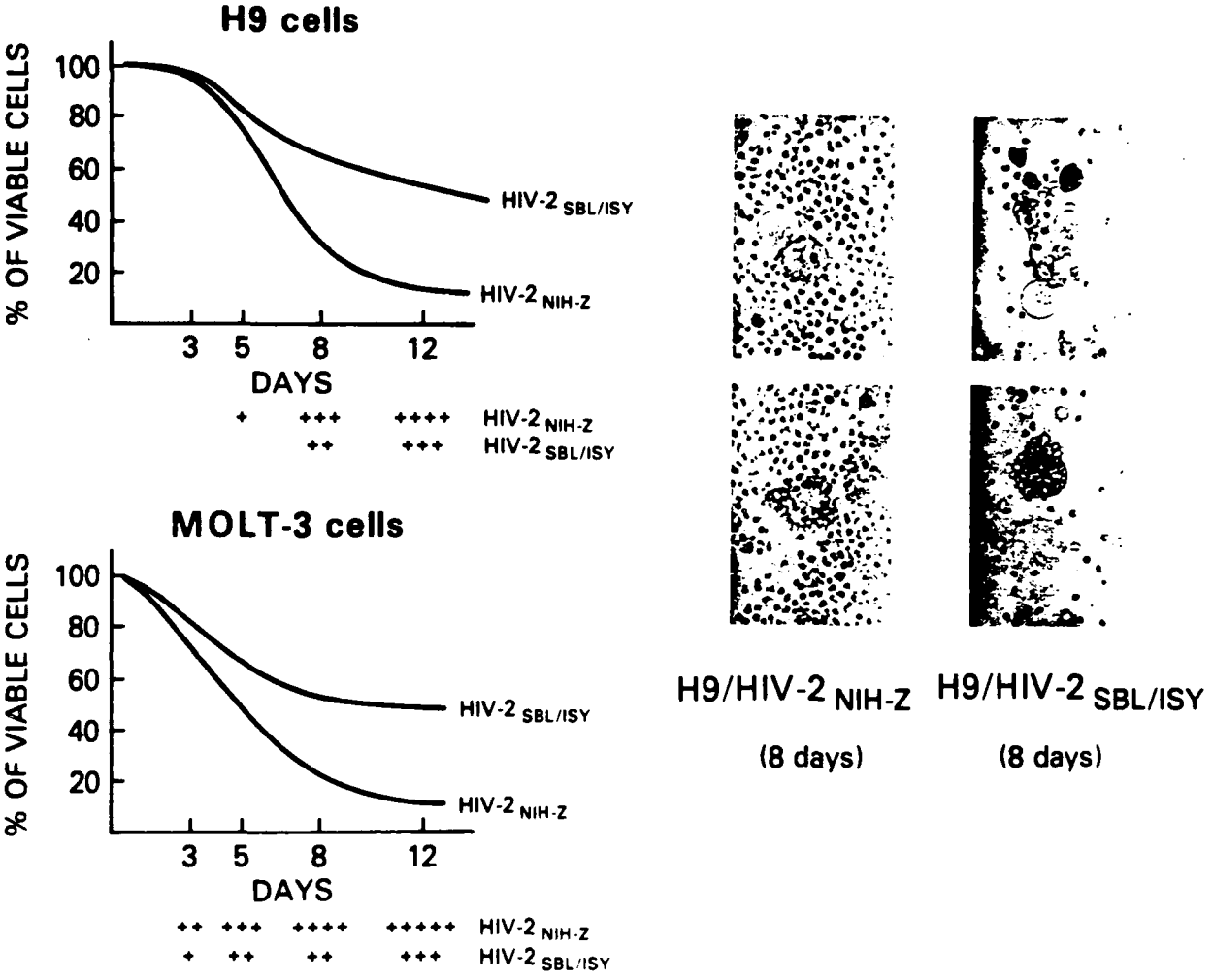


Figure 18: Cytopathic Effect of Human Immunodeficiency Viruses Type 2.

The left portion of the figure graphically represents the reduction in the number of viable cells in culture after infection with HIV-2_{NIH-Z} and HIV-$2_{SBL/ISY}$ isolates. The + symbol represent the relative scale of syncytia seen in cell culture at 3, 5, 8, and 12 days. An example of the size of syncytia seen at day 8 for both viral isolates is shown on the right side of the figure.

New Gene Name	Previous Name	MWT of Protein	Known Function
HIV			
tat	tat-3, TA	p14	transactivator of all viral proteins
rev (transregulator of expression of virion proteins)	art, ttrs	p19	regulates expression of virion proteins
vif (virion infectivity protein)	sor, A, P', Q	p23	determines virus infectivity
vpr (R)	R	?	Unknown
nef (retrovirus inhibitory factor)	3' orf, B, E', F	p27	Reduces virus expression, GTP-binding
vpx (X) (in HIV-2 and SIV)	X	p16 p14	Unknown

Table 1. Nomenclature of HIV Accessory Genes.

Construct	AA Changes in env	Replication RT/Cos-1 (Days)	Transmission H9 gag+	Molt-3 gag+
(Parental Clone - No Deletion)				
HXB2D	-00 +00	2+	+++	+++
(Mutants with Deletions in nef only)				
LR330	-00 +00	7+	+++	+++
(Mutants with Deletions in gp41 and nef)				
LR329	-00 +47	7+	++	++
LR206	-04 +59	2+	+++	+++
X10-1	-05 +15	2+	+++	+++
X9-3	-05 +76	7+	+++	+++
LR369	-06 +02	7+	+++	+
LR295	-14 +02	14+	+++	++
LR372	-15 +04	14+	+++	+
LR429	-15 +04	14+	++	-
LR269	-17 +02	14+	++	-
LR468	-33 +00	14+	++	-
LR362	-37 +18	14+	++	-
(Mutants with Deletions in the LTR/PPT as well as gp4l & nef)				
LR358	-06 +03	7+	-	-
LR318	-06 +04	7+	-	-
LR204	-06 +27	7+	-	-
LR312	-14 +74	7+	-	-
LR192	-17 +11	7+	-	-
LR189	-41 +00	7+	-	-
LR274	-42 +02	7+	-	-
LR194	-76 +09	7+	-	-
(Mutants with Deletions in tat/rev as well as above)				
LR360	-87 +32	-	-	-
LR327	-117+20	-	-	-

Key:
+ - < 20% positive in culture after four weeks
++ - >20% positive in culture in two weeks or less
+++ - >20% positive in culture in one week or less

Table 2. Replication and Transmission of C-Terminal env Deletion Mutants.
Cos-1 cells were transfected with pHXB2D (plasmid containing the HIV-1 genome) or deletion mutants of this plasmid. Virus replication was assyed by testing for reverse transcriptase activity in the spent supernatant of transfected cells at 2 days to 2 weeks post transfection. Viral transmission was assayed out by cocultivating polybrene treated H9 or Molt-3 cells with transfected cells for two days, separating the H9 or Molt-3 cells, and monitoring for the production of gag p24 protein by immunoflurescence of cells fixed in acetone at up to 4 weeks.

	H9		Molt-3	
Clone	TCID-50	GMT	TCID-50	GMT
pHXB2D	2435	1722	2048	1722
X10-1	430	609	181	431
LR468	49	50	54	38
LR429	45	32	87	70
LR362	4467	3158	790	790
LR269	3158	2896	558	558
LR372	4	4	ND	ND
LR32L9	108	83	470	664

Table 3: Titrations of C-Terminal env Deletion Mutants. Virus stocks were grown in H9 cells and concentrated 1000 fold by ultracentrifugation. Virus titrations were performed using serial dilutions in 20 µl RPMI 1640-20% fetal bovine serum. Four times 10^4 polybrene treated H9 or Molt-3 cells were added to each well and the incubation continued for 60 minutes at 37° C. Then 20 µl of culture from corresponding wells was transferred into duplicate microtiter plates containing 200 µl media. After 2 weeks incubation, infectivity was determined by the presence of gag p24 using an indirect immunofluorescence assay. Geometric mean titer (GMT) values and 50% infectious dose (TCID-50) were calculated using standard methods.

p24 Expression by IFA

Construct	AA Changes in env	H9 Week 1	H9 Week 3	Molt-3 Week 1	Molt-3 Week 3
pSV2Neo	NA	-	-	-	-
HXB2D	-0 + 0	65%	65%	65%	65%
LR318	-6 + 0	<1%	10%	-	<1%
LR204	-7 + 0	8%	68%	-	-
LR312	-14 + 0	-	<1%	-	-
RCLR274	-41 + 0	-	<1%	-	-
LR194	-75 +22	-	-	-	-

Table 4: Transmission by Mutants with Deletions Extending into tat/rev. Cos-1 cells were transfected with 5 μg of DNA and incubated with 5 x 10^5 polybrene treated H9 or Molt-3 cells. After 48 hours incubation, the cells were separated from the Cos-1 cells and maintained in RPMI 1640-10% FCS medium. At 1 and 3 weeks, the cells were applied to microscope slides, fixed with acetone, and examined by immunofluorescence withe anti-gag p24 monoclonal antibody.

VIRUS	ATH8 (H9)	ATH8 (Molt3)	HPB-ALL (H9)	HPB-ALL (Molt3)
None	0	0	0	0
HXB2D	100	100	100	100
X10-1	0	4	0	31
X9-3	0	0	25	31
LR295	0	65	0	50
LR362	0	0	0	80

Table 5: Cytopathic Effects of C-terminal ENV Deletion Mutants. Infected cells (5×10^4) from the experiment in Table 4 were washed and added to 1×10^6 polybrene treated ATH8 or HPB-ALL cells. Cells were incubated in RPMI-20% Fetal bovine serum and viability was assessed at one week by exclusion dye trypan blue.

	Day 0	Day 7	Day 14	Day 21	Day 28
Δ S					
$1x10^5$	10%	30%	28%	16%	2%
$2x10^5$	20%	50%	23%	20%	2%
HXB2D					
$1x10^5$	10%	30%	2%	3.50%	9%
$2x10^5$	20%	40%	8.75%	25%	35%

Table 6: Kinetics of vif Mutant Transmission. Mitomycin-C treated Molt-3 cells were infected with virus from Cops-1 transfected cells (1×10^5 or 2×10^5) The percentage of cells expressing HIV p24 was examined at weekly intervals by the immunofluorescence assay.

Cell-Associated Versus Cell-Free HIV-1 Neutralization
Results Using Wild-Type and vif Defective Clones
Difference between Cell-Free and Cell-Associated Titers

Sera ----- / Virus	HS01	HS02	HS03	HS04	HS05	HS06	HS07	HS08	HSB09	HS10	HS11	HS12	HS13
	Reciprocal Geometric Mean.50% Neutralization Titer												
III	16	1	16	1	16	256	1	1	16	16	64	1024	1
HXB2D	4	1	64	1	1	64	16	1	4	4	64	4096	1
DA4/Xgpt	80	320	20	20	20	5	1	1	20	20	5	20	5
AC4/3.3	1	20	20	5	1	1	1	1	20	20	5	5	1
AEC1/153	5	80	5	5	1	5	1	1	20	80	5	5	1

	HS14	HS15	HS16	HS17	HS18	HS19	HS20	HS21	HSB22	HS23	HS24	HS25	HS26
III	1024	64	4	4096	256	1	256	4	1	64	1	4096	4096
HXB2D	64	16	16	16	64	1	64	4	1	16	16	64	64
DA4/Xgpt	5	5	20	1	5	5	1	1	20	20	20	80	1
AC4/3.3	1	5	5	1	5	1	1	1	1	20	20	1	1
AEC1/153	1	1	20	1	1	1	1	1	5	80	5	1	1

Table 7. Cell-associated Versus Cell-Free Neutralization of Wild Type vif Defective Mutants of HIV-1

H9 cells were infected with 4 times the TCID50 of HXB2D, HTLV-IIIB (III) virus or cocultured with cloned cell lines infected with vif mutant viruses. The cells were treated with serial dilutions of various human sera positive for HIV to examine the ability of these sera to neutralize virus transmission. Expression of virus was examined at 2 weeks post infected by immunofluorescence with anti HIV-1 p24. The dilution of serum which gave a 50% reduction in virus titer is expressed as a reciprocal geometric mean titer.

Monkey and Human Sera

Reactivity to the Synthetic Peptides (PT-1, PT-2)

Species	Number Tested	Number Infected/Uninfected (Western Blot)	Number of Animals Reactive to PT-1 and PT-2
African green monkeys	17	17/0	0
Tala poin monkeys	6	3/3	0
Macaques (Experimentally infected)	6	3/3	2
Baboons (Experimentally infected)	3	3/3	0
Humans	7	5/2	0

Table 8. Monkey and Human Sera Reactivity to the Synthetic Peptides (PT-1 and PT-2)

Only 2 macaques out of 3 experimentally infected monkeys were reactive to peptides PT-1 and PT-2. None of the other monkeys or humans were reactive against these peptides.

		Days in Culture		
		5	8	12
MT-2	% IFA positive	ND	20	32
	RT	16	16	29
	Synctia	+	++	++
	% Viable cells	80	61	47
CL55	% IFA positive	ND	10	25
	RT	6	28	14
	Synctia	+	++	+++
	% Viable cells	70	40	36
CEM	% IFA positive	ND	4	18
	RT	7	9	12
	Synctia	-	+	+
	% Viable cells	98	76	71
HUT78	% IFA positive	2	4	18
	RT	13	10	36
	Synctia	-	+	++
	% Viable cells	90	71	50
U937	% IFA positive	3	ND	12
	RT	8	76	41
	Synctia	-	ND	ND
	% Viable cells	85	78	71
H9	% IFA positive	ND	10	20
	RT	18	ND	90
	Synctia	+	++	+++
	% Viable cells	83	63	41
MOLT 3	% IFA positive	ND	8	15
	RT	9	18	49
	Synctia	+	++	+++
	% Viable cells	77	53	43

IFA = immunofluorescence assay
RT = cpm x 10^3
Syncytia = +
% Viable cells = percentage of cells that do not incorporate Trypan blue

Table 9. Infectivity of the HIV-$2_{SBL/ISY}$ Isolate

HIV-$2_{SBL/ISY}$ infected the HTLV-I transformed T-cell line MT-2, the T-cell clone 55 immortalized line, and the CEM, Hut 78, Molt 3, H9, and U939 neoplastic cell lines. The highest cytopathic effect were observed in the HTLV-I infected cells and in the H9 cells.

		Days in Culture			
		4	7	11	14
Jurkat	RT	13	12	70	213
	Synctia	+	++	+++	+++
HUT78	RT	3	20	176	155
	Synctia	+	+	+	+++
U937-16	RT	17	8	80	279
	Synctia				

RT = cpm x 10^3

Syncytia = +

Table 10. Infectivity of the HIV-$2_{SBL6669}$ Viral Isolate

The parental virus HIV-$2_{SBL6669}$ infects Hut 78, U937 clone 16, and Jurkat T-cells. The highest cytopathic effect is observed on the Jurkat and U937-16 cell lines.

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1. Franchini, G., Kanki, P.J., Bosch, M., Fargnoli, K., Wong-Staal, F.1988. The simian immunodeficiency virus envelope open reading frame located after the termination codon is expressed in vivo in infected animals. AIDS Res. Human Retroviruses 4:251.

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